THE EFFICACY OF SELECTED PLANT EXTRACTS AGAINST ASPERGILLUS FLAVUS AND SITOPHILUS ZEAMAS ON POST-HARVEST MANAGEMENT OF MAIZE

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A THESIS SUBMITTED IN PARTIAL FULFILMENT FOR THE REQUIREMENT OF THE DEGREE OF MASTER OF SCIENCE (PLANT PATHOLOGY) IN THE SCHOOL OF PURE AND APPLIED SCIENCES OF KENYATTA UNIVERSITY

OCTOBER, 2017
DECLARATION

I declare that this thesis is my original work and has not been presented for award of a degree in any other university or for any other award.

Signature………………………… Date…………………………

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Declaration by supervisors

We confirm that the work reported in this thesis was carried out by the candidate under our supervision as university supervisors.

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DEDICATION

To my wife Mary, our children Olive and Austin for their unrelenting support and inspiration.
ACKNOWLEDGEMENT

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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>ACIAR</td>
<td>Australian Centre for International Research</td>
</tr>
<tr>
<td>ACS</td>
<td>American Chemistry Society</td>
</tr>
<tr>
<td>AFB1</td>
<td>Aflatoxin type B 1</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
</tr>
<tr>
<td>BCG</td>
<td>Bacillus Calmette-Guerin</td>
</tr>
<tr>
<td>Bt</td>
<td><em>Bacillus thuringiensis</em></td>
</tr>
<tr>
<td>CDC</td>
<td>Centre for Disease Control</td>
</tr>
<tr>
<td>CRC</td>
<td>California Resource Corporation</td>
</tr>
<tr>
<td>DC</td>
<td>District of Columbia</td>
</tr>
<tr>
<td>FAO</td>
<td>Food Agricultural Organization</td>
</tr>
<tr>
<td>GC-MC</td>
<td>Gas chromatography-mass spectroscopy</td>
</tr>
<tr>
<td>GM</td>
<td>Genetically modified</td>
</tr>
<tr>
<td>Hr</td>
<td>Hour</td>
</tr>
<tr>
<td>ICIPE</td>
<td>International Centre for Insect Physiology and Ecology</td>
</tr>
<tr>
<td>ICRAF</td>
<td>International Council for Research in Agro forestry</td>
</tr>
<tr>
<td>IDRC</td>
<td>International Development Research Centre</td>
</tr>
<tr>
<td>ISB</td>
<td>Indian School of Business</td>
</tr>
<tr>
<td>KARI</td>
<td>Kenya Agricultural Research Institute</td>
</tr>
<tr>
<td>Kg</td>
<td>Kilogram</td>
</tr>
<tr>
<td>LD&lt;sub&gt;50&lt;/sub&gt;</td>
<td>Concentration level that kills half of insect pest</td>
</tr>
<tr>
<td>LGB</td>
<td>Larger grain borer</td>
</tr>
<tr>
<td>M</td>
<td>Metre</td>
</tr>
<tr>
<td>Mg</td>
<td>Milligram</td>
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<tr>
<td>Ml</td>
<td>Mililitre</td>
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</table>
Mm  Milimetre
MMWR  Morbidity and Mortality Weekly Report
NACOSTI  National Commission for Science, Technology and Innovation
NSW  New South Wales
OICI  Opportunities Industrialization Centre International
PDA  Potato Dextrose Agar
Ppb  Parts per billion
Ppm  Parts per million
RSCU  Relative Synonymous Codon Usage
SEM  Standard error of mean
SIDA  Swedish International Development Agency
μl  Microlitre
USA  United States of America
USDA  United States Department of Agriculture
WHO  World Health Organization
WMO  World Meteorological Organization
ABSTRACT

Maize (*Zea mays* L.) is the most important cereal crop in Kenya with 90% of the total population relying on it as the main staple food. Post-harvest losses in maize is caused by insect pests such as *Sitophilus zeamais* and Fungi such as *Aspergillus* and *Fusarium* among others. Post harvest losses by insect pests can sometimes be up to 90 percent. Synthetic chemicals are used to manage weevil infestation and control mould development in maize. However, chemical residues have been detected on the stored maize prior to consumption. Additionally, continuous uses of synthetic chemicals have led to development of pathogen/pest resistance reducing their effectiveness. This study therefore sought to evaluate a possible use of *Ocimum kilimandscharicum* essential oil to manage both *Aspergillus flavus* and *Sitophilus zeamais*. The study also determined the effect of pretreating baglets with aqueous extracts of *A. indica* and *W. ugandensis* to enhance efficacy of *O. kilimandscharicum* oil. *Aspergillus flavus* was isolated from maize samples using direct plating method, identified and pathogenicity tests done. Laboratory experiments were carried out to establish antimicrobial and insecticidal property of *O. kilimandscharicum* oil. Additionally on farm experiments were carried out to establish the oil’s effectiveness and longevity. Maize grains were treated with *O. kilimandscharicum* oil in the first experiment and in the second treated maize grains were put in miniature synthetic bags treated with aqueous extracts of *A. indica* and *W. ugandensis* and stored in a granary for six months. *Ocimum kilimandscharicum* oil inhibited growth of *A. flavus* on petri dishes with concentration level above 100μl/ml of the essential oil having total inhibition. The size of zone of inhibition using disc diffusion method was significantly largest at 400μl/ml concentration level and lowest at 50μl/ml concentration level. However, the inhibition zones were significantly (p<0.0001) higher than in the control treatment. On-farm experiment revealed that *O. kilimandscharicum* oil doses were effective against *S. zeamais* as compared with untreated maize grains. However, 10 ml per 1kg of maize grains was the best dosage. Weight loss in maize grains was proportional to the number of holed grains. Replenishment of the oil after the third month (at 6 ml, 8 ml and 10 ml) reduced significantly (p<0.0001) the number of holed grains as well as weight loss. Pretreatment of baglets containing maize grains with aqueous extracts of *A. indica* and *W. ugandensis* significantly improved protection of maize grains treated with *O. kilimandscharicum* oil. From the results of this study it can be concluded that plant extracts can offer a possible substitute to synthetic chemicals in post harvest management of *A. flavus* and *S. zeamais* in stored maize. Use of essential oil of *O. kilimandscharicum* for post-harvest protection of maize grains against *S. zeamais* is therefore recommended.
CHAPTER ONE
INTRODUCTION

1.1 Background information of the study

Maize or corn (Zea mays) is a grass plant which belongs to the Family Poaceae. It grows to a height of up to four metres. The ears develop in leaf axils on the stalk, which terminates the tassel. The broad leaf sheath overlaps around the stalk and the leaves are arranged in two opposing row along the stalk. It is one of the most vital grain crops worldwide. The Mexican highland in the Mesoamerican region is believed to be its centre of origin, from where it spread rapidly (USDA, 2005). Archaeological records and phylogenetic analysis suggest that domestication began at 6,000 years ago (Piperno and Flannery, 2001; Matsuoka et al., 2002). It is documented that after the discovery of the Americas by Europeans, maize spread to the temperate zones in the 15th century (Paliwal, 2000; Farham et al., 2003).

Maize is a basic human food, but is also used as an animal feed as well as raw materials for production of many industrial products. Maize gives more carbohydrates compared to wheat and sorghum, and it is an important source of phosphorous (Brandes, 1992). Maize contains small amounts minerals such as Calcium and iron, vitamins such as thiamine and niacin, as well as small amounts of fat (Adeyemo, 1984). Furthermore, maize crop is important in ensuring food security to consumers since it gives more yields per unit area of land (Brandes, 1992). It is a very basic human food in Kenya, grown in almost all agro-ecological zones and on two out of every three farms. It accounts for nearly half of calories consumed (Smale et al., 2011) and has per capita consumption of 94 kilograms; this translates to between
30 and 34 million bags (2.7 to 3.1 million metric tons) of annual maize consumption in Kenya (FAOSTAT, 2010). According to Nyoro et al. (1999), Kenya imports maize from neighboring countries to bridge the gap since it produces only 28 million bags. Maize accounts for roughly 20 percent of gross farm output for the small-scale farming sector and is significant in Kenya’s crop production patterns (Jayne et al., 2001). It also is grown for subsistence, dual and commercial purposes.

Maize production in Kenya and other Sub-Saharan countries is hampered by poor weather, inadequate absorption of modern technologies such as high yielding varieties and fertilizers, lack of access to credit and inadequate extension services to small scale farmers (Kangethe, 2004). Furthermore, Poor infrastructure, insufficient budgetary allocations to agricultural development and the private sector’s in maize marketing in liberalized market (Republic of Kenya, 2008). Diseases, pests’ infestation and low soil fertility are other factors that hinder maize production (ICIPE, 2000).

Insect damage grain during grain storage and maize weevils (Sitophilus zeamais) are one of the most serious pests infesting maturing cobs as well as stored maize (Goodyer, 1995; Collins, 1998). Other stored cereal grains are also infested acting as alternative pest hosts. Wheat is among its notable secondary hosts that have become one of the basic human foods in Africa that help to alleviate protein deficiency and malnutrition young children.

Synthetic chemicals such as phosphine, carbon disulphide, malation, carbarlyl or permethin are used as fumigants that adversely subdue the destructive nature of entomons and other storage insect pests. These fumigants have been documented to
effectively combat the destructive activities of stored products pests (Ogunwolu and Idowu, 1994; Adedire et al., 2011). The usual fumigation methods are being re-evaluated in the developed countries due to various reasons such as depletion of the O-zone layer and ability of phosphine and methyl bromide to cause cancer (Adedire, 2002; Adedire et al., 2011). Lack of application knowledge, resistance by pests, great application cost, insects developing genetic resistance, harm beneficial insects as well as being harmful to the user directly, are some of the concerns linked to many synthetic insecticides (Okonkwo and Okoye, 1996; Akinkurolere et al., 2006; Oni and Ileke, 2008).

Uses of safer, cheaper as well as eco-friendly methods of managing pests attack in stored products are gaining popularity within the tropics (Lale, 1992). Use of edible plant materials as grain protectants are being given great attention currently (Adedire and Lajide, 2003; Akinkurolere et al., 2009; Adedire et al., 2011) and some of these plant species of which are also used for medicinal purposes are in great abundance in tropics.

Filamentous fungi produce secondary metabolites (mycotoxins) which are low molecular weight natural compounds harmful to all groups of animals even in very low quantities (Bernnet and Klich, 2003). The fungi that are associated with them normally grow on agricultural products such as cereals, grains, nuts as well as legumes prior and after harvest, during transportation or storage (FAO, 1998). Maize has one of the most serious mycotoxins problems of all crops (Munkvold, 2003). Common mycotoxins occurring in maize are aflatoxin produced by *Aspergillus flavus*, *A. parasiticus*; ochratoxin by *A. ochraceus, A. niger*; fumonisin by *Fusarium*
verticilliodes; trichothecenes by *F. graminearum*; zearalenone by *F. graminearum* (Blaney, 2004; Farrell and O’Keefe, 2007).

Natural plant extracts may provide an alternative way to protect stored maize from fungal and insect damage. This study therefore sought to evaluate the possibility of *O. kilimandscharicum* oil, aqueous extract of *A. indica* and *W. ugandensis* for post harvest management of both *A. flavus* and *S. zeamais*.

1.2 **Statement of the problem**

Maize is a basic human food in Kenya and other parts of Africa. It is cultivated in almost all agro-ecological zones and small scale holders’ accounts for 75 per cent of the total maize production while large scale farmers’ accounts for 25 per cent (Republic of Kenya, 2004). However, post harvest losses have hampered maize production. This is partly attributed to attack by fungal pathogens such *Fusarium moniliforme* and *Aspergillus flavus* which produce mycotoxins and the insect pests such *S. zeamais*, poor storage methods amongst others (Songa, 2004). The use of synthetic chemicals to manage weevils has in most instances led to development of pesticide resistance. High and acute toxicity, long degradation period, environmental pollution and carcinogenic concerns are some of the problems associated with synthetic chemicals used to control postharvest diseases.

Control failures and resistance to phosphine are very high in India and Australia (Rajashekar *et al.*, 2006). Ozone depletion has been associated with methyl bromide (WMO, 1995) and has been banned in developed countries. Malathion, chlorpyrifos, or deltamethrin are contact insecticides which offer protection to stored products insect infestation for several months. However, resistance by insect pests has been a
growing concern (Georghiou, 1990). Furthermore, adverse effects on food, side effect on human, residual toxicity and environmental pollution has been reported (Dubey et al., 2007; Kumar et al., 2007). The continuous and indiscriminate use of these insecticides has also led to development of resistant strains and accumulation of toxic residues on food grains for human consumption leading to health concerns (Sharma and Meshram, 2007).

1.3 Justification

Due to these problems associated with synthetic insecticides worldwide, alternative strategies for the development of newer insecticides that give almost different standards have been on the rise (Heyde et al., 1984; Dayan et al., 2009). These standards includes: made of locally available materials, less expensive, less susceptible to insect resistance, environmentally friendly, non toxic to humans, non phototoxic and pest specific (Hermawan et al., 1997). This has therefore led to re-examination of old methods of using plant derivatives to protect stored products from insect pests (Lale, 1992; Ewete et al., 1996; Talukder, 2006; Sahayaraj, 2008). This study sought to evaluate the efficacy of *O. kilimandscharicum* oil, aqueous extracts of *A. indica* and *W. ugandensis* for post- harvest management of *Aspergillus flavus* and maize weevils on maize.

1.4 Hypotheses

i. The essential oil of *O. kilimandscharicum* does not inhibit growth of *A. flavus* on petri dishes

ii. The essential oil of *O. kilimandscharicum* has no insecticidal property against *S. zeamais* on artificially infested maize grains in the laboratory.
iii. The essential oil of *O. kilimandscharicum* has no long-term protection on stored maize grains against *S. zeamais*.

iv. Pre-treatment of baglets for maize with aqueous extracts of *A. indica* and *W. ugandensis* cannot contribute to improved protection on stored maize grains against *S. zeamais*.

1.5 Objectives

1.5.1 General objective

To evaluate efficacy of selected plant extracts against *A. flavus* and *S. zeamais* on post harvest management on maize.

1.5.2 Specific objectives

i. To determine the effect of essential oil of *O. kilimandscharicum* on growth of *A. flavus*.

ii. To establish insecticidal property of essential oil *O. kilimandscharicum* against *S. zeamais* on artificially infested maize grains in the laboratory.

iii. To determine long-term protective ability of essential oil of *O. kilimandscharicum* against *S. zeamais* on stored maize grains.

iv. To establish possibility of improved activity of essential oil *O. kilimandscharicum* against *S. zeamais* on pre-treatment of baglets for maize grains with aqueous extracts of *A. indica* and *W. ugandensis*. 
1.6 Significance of the study

The study was carried out to determine the possible use of *O. kilimandscharicum* oil, *A. indica* and *W. ugandensis* extracts to manage postharvest losses in maize. The study evaluated whether the oil and the extracts could further be used both as antimicrobial and insecticidal as opposed to synthetic chemicals. The recommendations from the findings form the basis for further research on commercial use of the plant extracts in management of post-harvest losses in maize.
CHAPTER TWO
LITERATURE REVIEW

2.1 Origin of maize

Maize to belong *Zea* genus in Andropogoneae tribe, Panicoideae subfamily and in the Family Poaceae (USDA, 2005). The genus *Zea* in the tribe Andropogoneae presently have 86 recognized genera and five species (USDA, 2005). *Zea mays* is the only cultivated species; other species and subspecies are wild grass, referred to as teosintes (Doebley, 1990).

During maize domestication, a selection of maize cultivars or land races has been produced in every region in which it has been cultivated over the centuries. Farmers have maintained and made improvement on these and are adapted depending on requirements and characteristics of the local regions (Paliwal, 2000).

2.2 Production of maize

Farnham *et al.*, (2003) documented that maize can be grown in a variety of environments from 58° North to 40° South. In General, maize in tropical regions is grown between 30° North and 30° South, in subtropical regions ranges between 300 and 34° both north and south and in temperate regions, maize is grown beyond 34° latitudes. Maize is cultivated in altitudes ranging from sea level to 3,800 metres above sea level and grown between 42 and 400 days. Warm daytime temperatures should range from 25°C to 30°C and nights which are cool (Colless, 1992). Temperatures below 8°C or above 40°C usually cause cessation in maize development (Birch *et al.*, 2003). Different optimal temperature requirements are required for different maize cultivars. Tropical and sub tropical cultivars are more prone to water logging at initial
stage of growth and at ‘knee high’ stage (Srinivasan et al., 2004). Maize is categorized as being salt sensitive or moderately salt-tolerant plant (Kaddah and Ghowail, 1964; Lafitte, 2000) although cultivars respond differently to variation in salinity (Rao and McNeilly, 1999).

There are varied agro-ecological zones in Kenya in which different varieties are grown and these include hybrids for high altitude of over 1800M above sea level, those for medium altitude of 1000-1700M above sea level and those for low altitude of less than 1000M above sea level (Kenya Seed Company, 2012). A pH of 5.5-7.8 is suitable for growing maize. The effects of pH outside this range usually make certain minerals are not readily available. Rainfall requirement for highland varieties ranges from 800-1500 mm while those in medium altitude ranges from 750-1000 mm. Maize varieties in lowland zones require rainfall of about 400 mm. Varieties in dry agro-ecological zones ranges from 250 mm-500 mm (Kenya Seed Company, 2012).

In the year 2013, United States of America produced 32.1% of the total world maize production. China produced approximately 24.4%, Brazil accounted for 8.3% and Europe Union nearly 6.4% (USDA, 2013).

2.3 Uses of maize

Maize at various developmental stages can be consumed directly as food, from baby corn to mature grains. Stock feed takes great proportion, for example 40% in tropical countries and in developed countries up to 85% (Paliwal, 2000; Farnharm et al., 2003). It can be fed to livestock as green chop, dry foliage, silage or grain. A variety of food and drinks can be processed from. According to White (1994) maize is used as
food ingredient, either when modified chemically or in its natural form and is the major source of starch worldwide. Maize starch can be processed into alcohol, including fuel ethanol, while the largest non-food consumer of maize starch is the paper industry. The by-products of starch production: oil and protein are often of commercial value and are used in manufacturing industry (Boyer and Hannnah, 1994; Hobbs, 2003; McCutcheon, 2007).

Maize is one of the most important crops for human and has a high calorie feed for livestock (FAO, 1992). In humans, it’s an important source of vitamins such as B1, B2, C, and folate, minerals such as Phosphorous and Manganese as well as dietary fibre. It is similar in energy as dried legumes and compares favourably as staple with root and tuber crops (Okoruwa and Kling, 1996).

In Kenya maize is commonly used as food, fermented to produce a variety of food and beverages as well as livestock feed, Industrial input of starch, oil, sugar, protein cellulose and ethyl alcohol (Morris, 1998). Stalks, leaves and tassel are used as fuel or livestock feed either green or dried.

2.4 Insect pests as constraints to maize production

Maize is most prone to insects’ damage during early stages of growth and soil insects can pose up to 30% losses from tasseling to harvesting (Goodyer, 1995; Farrell and O’Keeffe, 2007; O’Gara, 2007). Pests that attack maize during development to maturity include Spodoptera exempta, helicoverpa zea, Cicadulina mbila, Monoptera australis, Rhopalosiphum maidis, Sitophilus zeamais and Frankliniela occidentalis (O’Gara, 2007).
Cereals are prone to insect infestation during grain storage, for instance maize weevil (*Sitophilus zeamais* Motschulsky (Coleoptera: Curculionidae)) is one of the most serious pest of stored maize and also attacking maturing cobs (Goodyer, 1995; Collins, 1998). In Africa, small-scale holders constitute the bulk of farmers growing maize for human consumption and animal feed. The world’s growing population experiences shortage of available arable land for agricultural production. Emphasis is on the relative importance of plant breeding to raise crop yield potential that adapt to the prevailing environmental conditions in the wake of climate change (Slater and Araus, 2007).

Post-harvest storage insect pests cause serious damage to cereals and in most cases make the stored grains prone to secondary attack by disease causing micro-organisms (Tefera *et al*., 2010). In tropics, grain losses lead to food insecurity and low farm incomes where greater losses are contributed by extreme temperatures and relative humidity (Azu, 2002). Most small-scale holders sell their cereals at throw away price to avert the likelihood of losses during storage and there after buy food at higher prices.

In Kenya maize is grown by nearly 90% of all farms. The significance of this to its economy ranges food provision, income generation and creation of job opportunities both directly and indirectly (Pearson *et al*., 1995). Post harvest losses due to insect pests have been estimated at 1.8 million (90 Kg) bags of maize estimated at 8.1 billion Kenya shillings annually. Existing pest management practices such as chemical control (Muhuru and Kibata, 1985; Kibata *et al*., 2003) and biological control (Giles *et al*., 1996) have had little impact in ameliorating storage losses.
The rise of insecticide use in farming has contributed to various problems such as the buildup of resistance by insects, environmental pollution, and out breaks of secondary pests (Chadwick and Marsh, 1993; Kortenhoff, 1993; Pimentel, 2002). In addition, careless uses of these chemicals cause poisoning of 3 million human beings a year. Synthetic chemicals have been widely used in storage facilities to control stored products from insect pests’ since the 1950s. Methyl bromide, phosphine, cyanogens, ethyl formate, or sulfuryl fluoride are fumigants that eradicate all life stages of stored product insects in a storage structure or in a commodity. Fumigation is one of the most effective methods used to eradicate insect pests and therefore prevent losses of stored products. However, there is development of pest resistance on stored products and a slight rise in resistance to fumigation.

2.4.1 Maize weevils (Sitophilus zeamais)

The maize weevil, *Sitophilus zeamais* Motschulsky (Coleoptera: Curculionidae) is one of the most serious post-harvest insect pests in the tropics. Loss in grain weight grain ranging 20–30% on average has been documented (Rees, 2004); and according to Boxall (2002); Tapondjou et al. (2000), up to 80% loss may result for untreated maize grain stored in traditional facilities depending on the storage period.

2.4.2 Biology of Sitophilus zeamais

Adult weevils occur on the maize fiels as maize attain roasting ear stage. Laying of eggs however, occur until the ear form. At this stage female weevil makes a small hole on the kernel in which the eggs are deposited. The female then seals the hole with mucilaginous material secreted by it (Hill, 2008). The eggs measures 0.7mm by
0.3mm, oval in shape and white in color. Upto five eggs per day may be laid and a total of 150 to 400 eggs in its life span (Bosque-perez, 1992).

Tiny grubs are hatched within four to nine days and larvae develop in about 25 days at about 30°C and relative humidity of about 70%. The grub has strong jaws, a brown head and white in colour. Pupation occur within the grain and last for about three to six days. Newly emerged adult remain in the grain for a few days before leaving (Chilio et al., 2004).

Sitophilus zeamais is a small weevil measuring 2.5mm to 4mm long with protruded snout, uniformly reddish brown or dark brown for boring and chewing grain. The elytra and prothorax are densely pitted with rows of microscopic circular holes. The wings are well developed for flying and have prominent legs (Kiritani, 1996).

2.4.3 Larger grain borer

The larger grain borer (LGB) (*Prostephanus truncatus*) is a post-harvest pest that has also led to losses of maize worldwide (Omondi et al., 2009; Tefera et al., 2010). The larger grain borer causes great grain losses by infesting on the grains and burrowing into them for oviposition. Grain losses arising from larger grain borer are approximated to vary from 9 - 45%, depending on the storage duration (Gueye et al., 2008; Kumar, 2002). The damage resulting from postharvest pests on the maize grain cannot be reversed and this affects all in the maize value chain including farmers, traders and consumers. To raise the quality and quantity of food grain that will feed the world’s increasing population, there is need to lower down losses from insect pests and diseases that infest maize in all stages of development and storage.
2.4.4 Mycotoxins

The major causes of food contamination world over are *Aspergillus* species (Adam *et al.*, 1998; Wangikar *et al.*, 2005). Aflatoxins-B1, B2, G1, G2 (produced by *A. flavus* and *A. parasiticus*), aspergillic acid and hydroxyaspergillic acid (produced by *A. flavus*), are some of mycotoxins produced by *Aspergillus* spp. in foods when subjected to suitable conditions (Leoni *et al.*, 2001; Marín *et al.*, 2004).

These toxins are among the most carcinogenic compounds known to cause serious problems all over the world in agricultural commodities such as maize and peanuts. Consumption of a mould-contaminated peanut meal was traced, isolated and characterized to be the cause of death of more than 100,000 turkey pouls due to the turkey X disease (Blout, 1961). Aflatoxins are both toxic and carcinogenic to human and animal populations (Eaton and Groopman, 1994).

The diseases caused by consumption of aflatoxin in human and animals are loosely called aflatoxicoses. Aflatoxicoses occur in two forms; acute aflatoxicosis that results in death and chronic aflatoxicosis that causes cancer, suppressed immune system, and other “slow” pathological conditions (Hsieh, 1988; Liu and Wu, 2010). The biggest documented aflatoxin poisoning outbreak happened in west of India in 1974, resulting in 106 deaths and 397 recognized cases and (Krishnamachari *et al.*, 1975).

Another outbreak of acute aflatoxicosis was reported in Makueni County, Eastern Kenya (Ngindu *et al.*, 1982; Probst *et al.*, 2007). In the former Eastern and Central provinces (Kenya) an acute hepatotoxicity outbreak was identified in April 2004 (CDC, 2004; Probst *et al.*, 2007). This was one of the largest and most serious
outbreaks of acute aflatoxicosis reported worldwide. The outbreak resulted in 317 cases and led to 125 deaths (CDC, 2004). Of the 317 case-patients, 89% resided in four Counties (Makueni, Kitui, Machakos, and Kiambu). Maize products (55%) in these regions had greater aflatoxin levels than the Kenyan acceptable limit of 20 ppb, 35% had levels greater than 100 ppb, and 7% had levels greater 1,000 ppb (CDC, 2004).

High and abnormal fluctuations in temperature and drought stress predispose pre harvest maize to fungal growth and subsequent mycotoxin accumulation. According to Chen et al. (2004), temperatures that facilitate growth of A. flavus are 17-42°C with aflatoxin production between 25-35°C. The amount of aflatoxin production and the fungus that inhabit them depend on moisture level of stored food products. Moisture content of 17.5% in stored grain is required for growth of A. flavus (Abbas, 2005). Mycotoxin contamination is also believed to be facilitated by infestation by insects which act as vectors for fungal spores and damage by insects result to wound in the plant through which fungal colonization occur (Munkvold, 2003). Genetically modified maize lines with Bacillus thuringiensis (Bt) in insect infested areas confer protection to plants from insect infestation and in turn have lower levels of mycotoxins than Non-Genetically Modified lines (Wu, 2008).

2.5 Insect pests and fungal control using plant extracts

Efforts to protect harvested produce against pests have been there since ancient times. The stored products were mixed with fire ashes by Egyptian and Indian farmers (Abdel –Gawad and Khatab, 1993; Varma and Dubey, 1999). Hassanali and Grainge (1986) reported that false hellebore (Veratrum album) was used as a rodenticide by
ancient Romans; the Chinese gets credit with discovering Derris species as having the insecticidal properties, whereas in China and Persia insecticidal property of pyrethrum was discovered. Locally available plants in many parts of the world are currently used to confer protection of stored products against insect infestations (Khater, 2012; Hassanali and Lwande, 1989; Akhtar et al., 2008; Tripathi et al., 2009).

According to Hassanali and Koppel (1985) neem leaves and seed are used by Indian farmers for the control of stored grain pests. Traditionally cowpeas are admixed with ash and then put into granaries or jars mud and clay in northern Cameroon (Wolfsen et al., 1991). Powel (1989) reported that Ocimum suave leaves and the cloves of Eugenia aromatic are traditionally used to protect stored grain in eastern Africa. In Rwanda farmers use a traditional closed structure (imboho) to store edible beans and to prevent insect attack within these structures. Ocimum canum whole leaves are usually added to the stored product (Weaver et al., 1991). Turmeric powder (2%) is traditionally mixed with rice or wheat stored in some south Asian countries (Saxena et al., 1988; Chatterjee et al., 1980). Oil of citronella, nicotine, derris, Pyrethrum and other plant extracts have been used as botanical insecticides for centuries (Sahayaraj, 2008; Sim et al., 2006; Singh and Upadhyay, 1993).

Natural plant extracts are proving to be more effective or safer alternatives to chemically produced antimicrobial agents and may offer a substitute method to prevent human food or animal feed from mould contamination (Thanaboripat, 2003). Extracts and powders of various spices, essential oils and herbs have been documented to have antimicrobial property and some also prevent aflatoxin formation.
(Bankole and Joda, 2004). Over the years, synthetic chemicals such as ammonia and propionic acid have been used to control stored grains against fungal attack have drawn considerable attention (Frazier and Westhoff, 1998). These compounds have been shown to be effective in preventing fungal growth. However, when they are too much on the grains there could be bring about chemical poisoning, environmental pollution and resistance development by fungi to the chemical agent.

Most natural products consumed by man, some which are tropical aromatic plants have been shown to exert high antimicrobial activities and there are little or no adverse effects even at very high doses (Adegoke et al., 2002). Thymus vulgaris (Nguefack et al., 2004), Cymbopogon citratus (Bankole et al., 2005) and Azadirachta indica (Bankole and Adebanjo, 1995) are some of these plants. Ocimum gratissimum L. has been exhibited to have variety of pharmaceutical uses (Ojeifo and Denton, 1993). Ocimum gratissimum leaves have reported to be a potential food preservative according to convincing in vitro evidence (Tagne et al., 2000). Furthermore, it has been shown to possess a high antifungal property against Aspergillus fumigatus, Aspergillus flavus and Fusarium moniliforme (Nguefack et al., 2004).

2.6 Plant extracts as antimicrobial and grain protectants

2.6.1 Antimicrobial and grain protectant properties of O. kilimandscharicum oil

The plants of the genus Ocimum L. (Lamiaceae) include: Ocimum kilimandscharicum, Ocimum gratissimum, Ocimum americanum, Ocimum campechianum, Ocimum sanctum and Ocimum basilicum. These plants are native throughout the world tropics and widely grown plants, escaped weeds and are recognized for their pharmaceutical potentials (Staples and Michael, 1999; Gupta et al., 2002). Extracts from plants of
Ocimum species have been documented regarding their essential oil’s antimicrobial activity (Kumar et al., 2010; Dambolina et al., 2010; Parkas et al., 2011).

*Ocimum kilimandscharicum* belongs to Lamiaceae Family and is an evergreen aromatic perennial shrub. The plant grows as a natural wood attaining a height of about two metres in warm temperate regions. Seeds and vegetative parts can be both used to propagate it. The plant has simple and opposite leaves with deeply serrated narrow bases (Warrier and Sala 1996). The leaves have aromatic oils, which are the essence of the plant. The essential oil is extracted through solvent extraction expression or distillation methods. The oil constitutes liquid oil and solid white crystals. The pure crystals have characteristic smell and taste of natural Camphor.

*Ocimum kilimandscharicum* extracts were traditionally used to cure various diseases in Eastern Africa such as diarrhoea, colds, measles, pains in abdomen, coughs, as a repellent to insects, especially mosquitoes and pests of stored products control (Kokwaro, 1976; Hassanali et al., 1990; Golob et al., 1999). One of its major constituents Camphor has been documented to be active against stored product beetles due to its protectant potential and toxicity (Ofori et al, 1998). It is classified as an aromatic plant whose bioactive constituents can find ready use in aroma therapeutic, pesticide industries and medicine according to research done on the insecticide and plant's medicinal efficacy (Bekele and Hassanali, 2000). The oil may be used as a solvent for metallic appearance on ceramic works due to its low boiling point.

Various researches have documented the antimicrobial property of essential oils on variety of food applications (Rasooli, 2008). Bioactive compounds present in essential
oils make essential oils a rich source of novel antimicrobial compounds of interest. Antibacterial, antifungal, antiviral, insecticidal and antioxidant are some of properties that essential oils were shown to constitute (Burt, 2004).

Obeng-Ofori et al. (1998) did the initial studies on the toxic effects of medicinal plants extract in the Institute for Stored Product Protection on O. kilimandscharicum, O. suave and O. kenyense. Camphor and eugenol were identified as main compounds of the essential oils depending on the Ocimum species. All three compounds were identified to be highly effective against beetles of the species Prostephanus truncatus, Tribolium castaneum, Lasioderma serricorne, Sitophilus zeamais and Sitophilus granarius if mixed with grain. Complete control was achieved after 24 hr at a dosage of 0.5 mg/kg or 0.5 μl/kg of grain. It was also proven that mixing of these compounds with low quantities of vegetable oils like sesame oil or sunflower seed oil increased persistency and toxicity to insects (Obeng-Ofori et al., 1998).

Compounds such as Camphene, 1, 8-cineole 4-terpeneol, limonene, α-terpineol, α-terpineol, endo-borneol, transcaryophyllene, camphor, myrtenol, and linalool are contained in aqueous extract of leaves of O. kilimandscharicum according to Eliningaya et al. (2009). Leaves also contain triterpenoids, saponins, tannins, sterols, flavonoids, carbohydrates and proteins (Paschapur et al., 2009). These chemical constituents are mainly responsible for various biological activities. Sethi et al. (2012) reported that camphor is the major component in O. kilimandscharicum oil while Gupta and Saxena (2010) documented that the oil possesses good to moderate antifungal activity. Antimicrobial activity against Gram-positive bacteria (S. aureus and Enterococcus faecalis), Gram-negative bacteria (E. coli, and P. aeruginosa) and
also against *C. albicans* yeast (Kumar *et al*., 2011) have been exhibited by essential oil from aerial parts of *O. kilimandscharicum*.

It has been documented that essential oils with anti-fungal effectiveness is able to constantly cause morphological changes in *Aspergillus* species including distortion of hyphae, aberrant development of conidiophores, failure of sporulation and loss of pigmentation, (Rasooli and Abyaneh, 2004). Various reports stated that the medicinal plants extracts play a significant role in inhibiting a variety of phytoxigenic fungi (Lin *et al*., 2001; Okemo *et al*., 2003; Choi *et al*., 2004; Khalil *et al*., 2005; Abd-El-Khair and Haggag, 2007; Perez-Sanchez *et al*., 2007). Natural bioactive materials contained in these extracts might have been responsible for inhibitory effect of tested extracts (Rasooli and Abyaneh, 2004).

Diterpenes, monoterpenes and hydrocarbons with various functional groups are the natural components present in essential oils. Antifungal and antimicrobial properties of essential oils have since been documented by many other researchers in pharmaceutical research and food applications, among others (Shittu *et al*., 2002; Benkeblia, 2004; Burt, 2004). However, little had been published previously on the use of essential oils as antifungal agents on food, feed and their products (Shittu *et al*., 2002; Burt, 2004).

### 2.6.2 Mode of action essential oils

The modes of action of essential oils remain somewhat controversial. Some studies indicate that the compounds may penetrate micro-organisms and react with active sites of the enzymes or interfere with cellular metabolism. However, more researches
indicate direct disruption of cellular membranes and concentration-dependent pro-oxidant cytotoxic effects (Bakkali et al., 2008).

Antifungal activities of essential oil involve penetration through cell walls and direct damage to both cytoplasmic and mitochondrial membranes (Bakkali et al., 2008). This result to change in permeability leading to leakage and finally cell death (Bakkali et al., 2008).

2.6.3 Safety of *O. kilimandschricum* extracts

The steam of leaves of *O. kilimandschricum* is used to treat serious colds in Kenya’s countrysides. For coughs and colds, leaves can be rubbed between palms and sniffed. Leaf water decoction can be drunk to alleviate abdominal pains and treat diarrhoea and measles in small children (Kokwaro, 1976).

2.6.4 *Azadirachta indica* extracts as grain protectant

Over many years, neem (*Azadirachta indica*) (A. Juss.), has come under close scrutiny scientifically as a potential source of botanical insecticide (Schmutterer, 2002). The tropical tree is neither infected nor infested by diseases and pest respectively. It is widely found in Africa and Asia.

Azadirachtin is one of the major constituent found the *Azadirachta indica* tree, cultivated in Africa and India (Isman, 2006). Azadirachtin is almost nontoxic to mammals and is least harmful commercial botanical insecticides, and has an LD₅₀ of 13,000 mg/kg. Azadirachtin exhibits “some” systemic property when applied on plants leaves but is also documented to be a contact poison. The compound is usually not toxic to insects and mites that are beneficial. Azadirachtin has a wide range of activity, acting a growth regulator in insects, repellent, feeding deterrent, inhibit
oviposition and sterilant (Rembold, 1989; Isman, 2006). The compound is also potent on a variety of insects, such as mealybugs, caterpillars, aphids and pests of stored products (Morgan, 2009).

Grain meant for storage admixed with dried neem leaves has been an ancient practice in India. The practice of admixing stored products with neem derivatives became part and parcel of traditional culture and wisdom in India. Pruthi and Singh (1944), documented that putting 5-7 inches thick layer of neem leaves in grains and crushing’s of neem fruits on the inner sides of grain containers was a common practice in India. Other common ways include making earthen bins with the mud mixed with neem leaf paste and overnight soaking of gunny bags in boiled neem leaf extract (2-10%), which were later used for storing grain. In Ghana, cacao remained free of *Ephestia cautella* infestation by up to 9 months in storage when beans were mixed with 8% neem leaves (Fry, 1938). Protection of stored products using neem in Nigeria tradition is well-documented (Prevett, 1962; Giles, 1964; Bugundu, 1970).

In Togo, Adhikary (1981) found that maize stored in sacks or unthreshed maize cobs kept in bins and treated with neem oil was effective against *Cathartus* spp., *Rhyzopertha dominica*, *Tribolium* spp. and *S. zeamais*. In Ghana, Tanzubil (1987) showed that neem oil at 0.5% admixed with cowpeas, or treated with 10% neem powdered fruit was effective against *Callosobranchus maculatus* up to 16 week storage; grain admixed with neem leaf dust was less effective, while 90% grain damage was observed in untreated cowpeas.

In India, neem kernel powder mixed sorghum seed in a proportion of 1.5 to 100 parts (wt/wt) was effective against *Sitophilus oryzae* infestation (Deshpande,
Neem oil (1%) was effective against attack by *S. oryzae* when maize seeds were soaked for 20 minutes (Attri and Prasad, 1980). A trial conducted in a warehouse in Philippines using neem oil alone or when combined with fumigation showed efficacy against five species of the major pests of stored products infesting paddy grains and rice (Jilani and Saxena; 1988). Neem oil at 0.05 to 0.1% mixed with rice grains or in combination with 'Phostoxin', and stored up to 8 months greatly reduced number of *Tribolium castaneum* adults than in the untreated control.

In Malaysia, paddy grain treated with neem leaves in a proportion of 2 to 100 parts (wt/wt), or putting neem leaves as barriers between bags and storage floor, immensely reduced infestation by *S. oryzae* and *R. dominica* (Muda, 1984). It was not established which treatment was better, but all treatments had the ability of being adopted in rural regions.

Radwaski (1981), reported that almost every part of *Azadirachta indica* A. Juss (seeds, fruits, flowers, leaves, bark, trunk and roots) is known to have some use in the regions where neem is found. The insecticidal activity of neem products on a variety of insect pests has been demonstrated by Shapiro *et al.* (1994). According to Shapiro *et al.* (1994), the derivatives reduce resistant development in insects, deter feeding and inhibit oviposition on plants, disrupt behaviour and physiology of insect in variety of ways and repel the approaching insects. Derivatives are eco-friendly and not harmful to human beings (Khattak *et al.*, 2001). Neem eliminates insect pests with soft bodies or their juveniles, although it is basically anti-ovipositional, insect growth regulator, feeding deterrent and repellent (Jacobson, 1988).
2.6.5 *Warburgia ugandensis* extracts as grain protectant

*Warburgia ugandensis* Sprague (Canellaceae) is a luxurious canopy level tree species of east Africa’s semi-deciduous and moist natural forests. The tree is important for furniture and timber for building, anti-bacterial and anti-fungal medicine (young twigs, bark and roots), food seasoning, resin, mulch, green manure, shade and ornamental (Mbuya *et al*., 1994; Katende *et al*., 1995). Seeds and leaves are added curries to add flavor and as livestock fodder while the fruits are eaten together with a hot peppery taste (ICRAF, 2009). Other products from this tree include insecticide, firewood, veterinary medicine, soap, toothbrushes, and charcoal (Maundu and Tengnas, 2005). The wood oil content is very high and ignites well with an incense-like smell while the gum/resin is used locally as glue to fix tool handles (Katende *et al*., 1995).

*W. ugandensis* is highly valued due its medicinal properties (Olila *et al*., 2002; Wamalwa *et al*., 2006). In Kenya detailed study of the tree species ranked it second as medicinal plant (Wamalwa *et al*., 2006). According to FAO (1986) and Wamalwa *et al*., (2006), dried bark of *W. ugandensis* is commonly used to alleviate ailments such as: toothache constipation, muscle pains, cough, fever, weak joints and stomachache and general body pains when chewed and the juice swallowed. Diarrhoea can be prevented by mixing soup with boiled fresh roots. Several skin diseases are cured through leaf decoction baths. Decoction from boiled bark, roots or leaves when drunk can be used to treat malaria (Katende *et al*., 1995; ICRAF, 2009). Muzigadial (a cytotoxic sesquiterpene) has been isolated from *W. ugandensis* and used to treat nagana (Olila *et al*., 2002) and other parasitic diseases in animals (Kioy *et al*., 1990).
Previous studies on *W. ugandensis* have documented that it possess good antiviral activity, antifungal, trypanocidal and antibacterial effects. Purified compounds and crude extracts of *W. ugandensis* have shown activity against *M. bovis* BCG Pasteur and *Mycobacterium tuberculosis* H37Rv (Madikane et al., 2007), measles virus and *Candida albicans* (Olila et al., 2001; 2002). The antimicrobial effects of *W. ugandensis* have previously been demonstrated. *Aspergillus niger*, *Alternaria passiflorae* and *Fusarium oxysporum* are soil pathogens in which *W. ugandensis* was found to be active against according to a study in Kenya (Rugutt et al., 2006), Olila et al. (2001), have documented that *W. ugandensis* has both antifungal and antibacterial activities.

*Warburgia ugandensis* and other *Warburgia* species have a terpenoid called Ugandensidial (Norris, 1986; Warthen & Morgan, 1990). It acts on *Spodoptera* species at 0.1 ppm (Warthen & Morgan, 1990) and twice as potent as azadirachtin (from neem oil) to *Spodoptera exempta*. According to Bekalo et al. (1996) warburganal, tannin, mannitol, polygodial, epipolygodial, ugandensidial, ugandensolide and muzigadial are some of the compounds (alkaloid group) found in *W. ugandensis* bark. Antiviral, antibacterial, fungicidal and trypanocidal activity are variety of biological effects that have been ascribed to the compounds.
CHAPTER THREE
MATERIALS AND METHODS

3.1 Description of the study area

The study was carried out in Murang’a County. The land rises gradually from an altitude of 914 M in the East to 3,353 M above sea level along the slopes of the Aberdares. The highest areas to the West have deeply dissected topography and are well drained by several rivers. The county experiences two rainfall seasons, March to May being the long rainy season and October to November which is the short rainy season. Areas with the highest potential have an average annual rainfall ranging between 1400 mm and 1600 mm. Low potential areas experiences rainfall less than 900mm annually. In medium and high potential areas rainfall is well distributed and reliable all round the year and is enough for agriculture. In low potential areas rainfall is not reliable and not well distributed and therefore cash crop farming is not suitable. Temperatures depend on altitude. In high altitudes temperatures can be as low as 6°C while in medium potential areas temperatures are moderate.

Land in the county is widely used depending on its wide Agro Ecological Zone from Tropical Alpine on the highlands to Lower Midland 4 on the low lands. Forested areas are on the highest grounds while cash crops like tea, coffee and macadamia follow the forest in that order. Maize and beans are grown in lower and mid zones of the county. Mathioya and Kangema sub counties border the forests and have a good climate suitable mainly for tea farming due to hilly and steep topography.
3.2 Collection of maize samples.

A total of eighty farms were sampled where maize had already been harvested and no synthetic chemicals had been initially dusted in the granaries, houses and other storage structures in Kangema subcounty. Samples of one kilogram were collected from these storage structures. Multiple sampling was done from 90 Kg bags or any other storage structure in the same granary and combined to constitute a sample of 1Kg.

Eighty kilograms of threshed maize were collected, dried over sun to moisture content of 13% which was determined using electronic moisture meter (Wile 55 type). The
sample was then divided into two, a quarter of the sample for mycological analysis and three quarters of the sample for insecticidal property of the oil.

3.3 Isolation of Aspergillus flavus from maize samples

Potato dextrose agar (PDA) was the standard media used to isolate the fungal pathogens from maize kernels. Maize kernels were plated on potato dextrose agar (PDA) medium by direct plating method. Further sampling was done to get two hundred maize kernels for mycological analysis. The grains were then surface sterilized with Sodium hypochlorite for 1 minute and twice rinsed with sterilized distilled water. The kernels were placed on sterile filter paper to dry. Samples were then plated on PDA plates at a rate of 5 kernels per plate (plate 3.1). The plates were incubated for 7 days at 25± 2°C. When the fungal cultures were established 1-2 cm from the maize kernels, sub-culturing onto fresh PDA media plates was done by cutting margin of the mycelia with a flame sterilized 5 mm cork borer.

Plate 3.1: Maize kernel directly plated on for isolation of A. flavus
3.4 Identification of fungi

Isolated fungi were further sub-cultured to obtain a pure culture. Identification was then done based on colony characteristics, morphology and microscopic features according to Klich (2002).

Fungal identification was done using morphological characteristics and comparing the findings with established keys as described by Bernnet and Hunter, (1999). Each isolate was subjected to colony and microscopic examinations during which their morphological features were observed and recorded. Morphological features studied were based on growth patterns, color of mycelia and microscopic examinations of vegetative and reproductive structures.

A sterile inoculating needle was used to get a small portion of mycelia from between the colony centre and the edge and placed on a clean microscopic slide containing lactophenol in cotton blue. The mycelia were spread well on the slide using the sterile needle and a cover slip gently placed with little pressure to eliminate air bubbles. The slide was placed above some boiling water to steam it for better staining of fungal structures. The excess lactophenol on the edges of the cover slip was wiped using sterile blotting paper. The slide was mounted on the microscope and observed with ×10 and ×40 objective lenses.

3.5 Pathogenicity test of A. flavus on maize grains

To determine whether the A. flavus infects maize grains, pathogenicity tests of identified fungi was performed in vitro according to Koch’s postulates. Kernels collected from the field were surface sterilized with sodium hypochlorite for five
minutes followed by thorough rinsing with sterile water. Five grains were blotted on sterile filter paper and thereafter inoculated by soaking them in a homogenized mycelia suspension of *A. flavus* (1×10⁶ spores). Five maize grains per replication were placed on PDA plates and subsequently incubated at 28±2°C for growth to check for fungal activity for 14 days. Control samples were treated with sterile water.

The inoculated healthy maize grains started to rot after the fourth day of inoculation. The grains were completely consumed with extensive mycelial growth forming a dark color covering the testa, (plate 3.2). The symptoms developed on inoculated maize kernels were compared with those of control experiment and results confirmed pathogenicity of the isolates on maize grains.

Plate 3.2: Maize grains inoculated with *A. flavus* (a) and negative control (b).
3.6 Collection of plant parts of *O. kilimandscharicum*, *A. indica* and *W. ugandensis* for oil and extracts samples

3.6.1 Sources of plant parts for oil extraction and aqueous extracts

*Ocimum kilimandscharicum* aerial plant parts were collected from wild population in Ruiru, Kiambu County. Taxonomic identification of the plant was done in Plant Sciences Herbarium in which voucher specimen was deposited (plate 3.3). One kilogram of both fresh *A. indica* and *W. ugandensis* leaves was collected from several trees at Kenyatta University medicinal garden (plate 3.4; plate 3.5).

![Plate 3.3: *O. kilimandscharicum* plant used in this study](image)

3.6.2 Extraction of oil from *O. kilimandscharicum*

The collected plant materials were air dried under shade in a well ventilated area for five days before extraction. The essential oils were extracted by hydro-distillation using Clevenger’s type apparatus for 4 hours at ICIPE (Nairobi). Extraction of the distillate by hexane was followed by drying over anhydrous sodium sulphate and the
removal of solvent using rotary evaporator. Oil collected was stored in air-tight bottles and kept in a refrigerator.

### 3.6.3 Extraction of A. indica and W. ugandensis extracts

The collected leaves were washed under tap water, rinsed three times in sterile distilled water and blotted dry by use of sterile blotting paper. The leaves were mixed with one litre of distilled water in a sterilized glass beaker, boiled for 2 hours to obtain water extract. The water extract was put in sterilized beakers after cooling.

Plate 3.4: *Warburgia ugandensis* tree at Kenyatta University medicinal garden.

Plate 3.5: Neem tree used in this study at Kenyatta university medicinal garden
3.7 *Antifungal activity of* *O. kilimandscharicum on A. flavus*

Antifungal assay was performed by agar disc diffusion and disc diffusion methods. Potato dextrose agar, with different amounts of essential oil (3.33 μl ml⁻¹, 6.67 μl ml⁻¹, 13.33 μl ml⁻¹, 20 μl ml⁻¹ and 26.67 μl ml⁻¹) was prepared by adding 400 μl, 300 μl, 200 μl, 100 μl and 50 μl of oil to 15 ml melted PDA each. Manual rotation was carried out to disperse oil in the medium and about 20 ml of the medium was poured into sterile petri dishes.

Each Petri dish was inoculated at the centre with mycelia disc (6 mm diameter) taken at the periphery of an *A. flavus* colony grown on PDA. Each PDA amended with *O. kilimandscharicum* oil was replicated four times and experiment arranged in completely randomized design. Control treatment comprised plain PDA. Plates were incubated at 25°C and colony diameter recorded for seven days. Growth inhibition was calculated by getting the mean colony diameter of the treatments and comparing with the control.

Using disc diffusion method, 5 mm filter paper discs were cut and then sterilized using an autoclave. * Ocimum kilimandscharicum* oil was prepared at different concentration by diluting 400 μl, 300 μl, 200 μl, 100 μl and 50 μl of oil with 1 ml of hexane (400 μl/ml, 300 μl/ml, 200 μl/ml, 100 μl/ml and 50 μl/ml). The sterilized filter paper discs were then immersed in different oil concentration for 30 minutes and then air dried. Filter paper discs were then carefully placed on solidified PDA medium amended with *A. flavus* spores suspension (1×10⁶ spores). Each treatment was replicated four times and experiment was arranged in completely randomized design. Control treatment comprised of sterile filter discs placed on PDA amended with *A.
*flavus* suspension. The size of zone of inhibition was determined at an interval of 2 days for one week.

3.7.1 Insecticidal property

*Ocimum kilimandscharicum* oil at different doses was applied on maize kernels with no sign of weevil infestation. Twenty (20) grams of maize kernels were further sampled and treated with 5μl, 15μl, 30μl, 45μl, 60 μl, 90 μl and 105 μl of oil which had been shaken with hexane to ensure uniform distribution of the oil over the grain surface. The control treatment comprised of untreated grains. Each treatment was replicated four times and arranged in a completely randomized block design. The maize grains were put in 50 ml glass jars and covered with cheese cloth fastened with a rubber band to allow aeration. Ten adult *Sitophilus zeamais* were introduced into each of the jars containing treated and untreated grains. The numbers of dead weevils were counted at an interval of 2 days for one week to determine *S. zeamais* mortality due to presence of *O. kilimandscharicum*. The weevil was considered dead when the limbs did not move when probed with a soft camel brush (Arannilewa et al., 2006).

3.8 On-farm experiments

3.8.1 Insecticidal activity of *O. kilimandscharicum* oil on *S. zeamais*

The insecticidal property of *O. kilimandscharicum* oil was further investigated on-farm in Kangema Subcounty to determine its effectiveness and longevity. Conventional insecticides such Pirimophos methyl (Actellic super™) remains effective for approximately six months on stored maize. The method of Mutambuki *et al.* (1999) was adopted with modification. The modification involved the use of miniature bags instead of 90kg gunny bags. The treatment included different amounts
of essential oils (2ml, 4ml, 6ml, 8ml and 10ml) dissolved in 1ml hexane and mixed with maize grains. The maize grains were left for one hour to allow the solvent to evaporate.

The treated maize grains were then put in small gunny bags (30cm by 10cm) and replicated four times (plate 3.6). Control treatments were set using untreated maize and Actellic super™ at the recommended dose of 50g/90kg as a positive control. The experiments were arranged in a completely randomized design. The small gunny bags with treatments and controls were put under the same conditions that would simulate those of a typical granary. *Sitophilus zeamais* infestation and percentage weight loss in the small bags was then checked monthly for six months. One hundred grams of the grains from each treatment replicate were sampled and analysed for infestation by weevils following the international count and weigh method described by FAO (1995).

Plate 3.6: Bags containing maize grain treated with different doses of *O. kilimandscharicum* oil and stored in granary for six months
In second experiment, one kilogram of maize grains was treated with *O. kilimandscharicum* oil at 6ml, 8ml and 10ml and after three months the same concentrations were replenished. The treatments were replicated four times with a control set using untreated maize grains. The experiments were then arranged in a completely randomized design. One hundred grams of the grains from each treatment replicate were sampled and analysed for infestation by weevils following the international count and weigh method described by FAO, (1995) for six months.

3.8.2 Pre-treatment of baglets containing maize treated with aqueous extracts of *A. indica*, *W. ugandensis*.

In the third experiment the baglets were soaked in 500ml of aqueous extracts of *A. indica* and *W. ugandensis* overnight and extracts allowed to dry. Six millilitre of *O. kilimandscharicum* oil was dissolved in one millilitre hexane, mixed with one kilogram maize grains and left for one hour for the solvent to evaporate. Maize grains were then put in the baglets and replicated four times (plate 3.7). Control treatments were set using untreated maize grains in unpre-treated baglets and treated maize in unpre-treated baglets. The experiments were arranged in completely randomized design. The baglets together with maize grains were kept in granary and infestation by *S. zeamais* was checked monthly for six months using international count and weigh method described by FAO, (1995).
Plate 3.7: Baglets treated with aqueous extracts of *A. indica* and *W. ugandensis* containing maize grains treated with *O. kilimandscharicum* oil.

### 3.9 Data analyses

Data on radial inhibition, number of holed grains and weight loss obtained were entered in a spread sheet, Microsoft Excel and normality determined. Transformation by logarithm, $\log_{10}(X+1)$ was done followed by two way ANOVA using SAS 9.0 software. The means were separated using Tukey’s HSD test at 5% level of significance. Results were finally presented in tables and graphs.
CHAPTER FOUR

RESULTS

4.1 *Aspergillus flavus* identification
The fungus isolated from maize grains grew on PDA with colonies that were yellow to dark, yellowish-green with a cream lower side; conidiophores appeared dense felt or mature vesicles bearing phialides over their entire surface (plate 4.1; plate 4.2). Texture was woolly to cottony and appeared granular. Sclerotia, were, dark brown to black. A clear to pale brown exudates was present in some isolates. Green colonies had rough conidiophores, with some appearing smooth to finely roughed conidia.

Microscopic characteristics of the fungi included long and septate cells borne on hyaline hyphae, colourless conidiophores that are wide and roughened, globose vesicles held on long conidiophores, and smooth globose conidia (plate 4.3). The identified fungi were maintained as pure culture on PDA for further testing and referencing in a refrigerator at 4°C.

Plate 4.1 Upper side  Plate 4.2 Lower side
*Aspergillus flavus* isolate on PDA showing upper and lower side of the colony in plate 4.1 and 4.2 respectively.
Plate 4.3 Microscopic conidiophores bearing conidia of *A. flavus*

**4.2 Efficacy of essential oil of *O. kilimandscharicum* on inhibiting growth of *A. flavus* on PDA in petri dishes.**

**4.2.1 Radial growth of *A. flavus* inhibition by *O. kilimandscharicum* oil by Agar disc diffusion**

The study revealed that the *O. kilimandscharicum* oil caused radial growth inhibition of *A. flavus* with varying concentration levels exhibiting varying effects. At higher concentrations of 26.67μl/ml, 20μl/ml, 13.33μl/ml, and 6.67 μl/ml radial inhibition of *Aspergillus* spp was 100% plate 4.4, plate 4.5, plate 4.6 and plate 4.7. At 3.33μl/ml concentration level radial inhibition was 43% (plate 4.8).

Plate 4.4 PDA amended with 26 μl/ml concentration level of *O. kilimandscharicum* and Plain PDA (control)
Plate 4.5 PDA amended with 20 μl/ml concentration level of *O. kilimandscharicum* and plain PDA (control)

Plate 4.6 PDA amended with 13.33 μl/ml concentration level of *O. kilimandscharicum* and Plain PDA (control)

Plate 4.7 PDA amended with 6.67 μl/ml concentration levels of *O. kilimandscharicum* and plain PDA (control)
Plate 4.8 PDA amended with 3.33 μl/ml concentration levels of *O. kilimandscharicum* and plain PDA and (control)

Radial inhibition of *Aspergillus* spp in 26.67μl/ml, 20μl/ml, 13.33μl/ml, and 6.67μl/ml concentration levels were not significantly different. However, the concentrations differed significantly (p<0.0001) with 3.33μl/ml and plain PDA (Table 4.1). Colony diameter (in mm) differed significantly (p<0.0001) in day 1, 3, 5 and 7. The results revealed that varying concentrations of *O. kilimandscharicum* oil significantly acted against *A. flavus*. Significant interaction effects (p<0.0001) were observed between concentration and days on colony diameter (figure 4.1).
Table 4.1: Mean colony diameter (in millimetres) of *A. flavus* on PDA amended with different concentration of *O. kilimandscharicum* oil

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Colony diameter</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plain PDA</td>
<td>1.26±0.06a</td>
</tr>
<tr>
<td>3.33μl/ml</td>
<td>1.07±0.40b</td>
</tr>
<tr>
<td>6.67μl/ml</td>
<td>0.69±0.001c</td>
</tr>
<tr>
<td>13.33μl/ml</td>
<td>0.69±0.001c</td>
</tr>
<tr>
<td>20μl/ml</td>
<td>0.69±0.001c</td>
</tr>
<tr>
<td>26.67μl/ml</td>
<td>0.69±0.001c</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Days</th>
<th>Colony diameter</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 1</td>
<td>0.69±0.001d</td>
</tr>
<tr>
<td>Day 3</td>
<td>0.75±0.20c</td>
</tr>
<tr>
<td>Day 5</td>
<td>0.81±0.50b</td>
</tr>
<tr>
<td>Day 7</td>
<td>0.83±0.60a</td>
</tr>
</tbody>
</table>

**P-values**

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Concentration</td>
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</tr>
<tr>
<td>Day</td>
<td>0.0001</td>
</tr>
<tr>
<td>Concentration*Day</td>
<td>0.0001</td>
</tr>
</tbody>
</table>

Values are expressed means ± SEM for four replicates. Means within respect columns followed by similar small case letters are not significantly different at P <0.0001 by Tukey’s HSD test.

![Figure 4.1 interactive effects of concentration of *O. kilimandscharicum* oil with number of days on colony diameter.](image-url)
4.3 Size of zone of inhibition of *A. flavus* grown on PDA by *O. kilimandscharicum*.

The study using disc diffusion method revealed similar results as agar disc diffusion method. At 400μl/ml concentration level the mean size of zone of inhibition was 7.08 mm on the seventh day (plate 4.9); at 300μl/ml concentration level (plate 4.10) the size of zone of inhibition was 6.53 mm while at 200μl/ml concentration level the size of zone of inhibition was 6.10 mm (plate 4.11). At 100μl/ml concentration level the size of zone of inhibition was 5.93 mm (plate 4.12). At 50μl/ml concentration size of zone of inhibition was least with 5.05 mm (plate 4.13).

Plate 4.9 Filter discs immersed in 400μl/ml concentration level of *O. kilimandscharicum* and control
Plate 4.10 Filter discs immersed in 300μl/ml concentration level of *O. kilimandscharicum* and control

Plate 4.11 Filter discs immersed in 200μl/ml concentration level of *O. kilimandscharicum* and control
Plate 4.12 Filter discs immersed in 100μl/ml concentration level of *O. kilimandscharicum* and control.

Plate 4.13 Filter discs immersed in 50μl/ml concentration level of *O. kilimandscharicum* and control.

The size of zone of inhibition was significantly larger (p<0.0001) at 400μl/ml concentration level of *O. kilimandscharicum* (Table 4.2) than all other concentration levels. The inhibition zones at 400μl/ml to 50 μl/ml range of concentration levels were significantly higher (p<0.0001) than in control treatment (Table 4.2). The size of zone of inhibition differed significantly (p<0.0001) from each other in day 1, 3, 5 and 7. Significant interaction effects (p<0.0001) were observed between concentrations and days on the size of zones of inhibition (figure 4.2).
Table 4.2: Mean size of zone of inhibition in millimetres of A. flavus culture grown on PDA and filter paper discs impregnated with *O. kilimandscharicum* oil

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Size of zone of inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plain PDA</td>
<td>5.00±0.00 (^f)</td>
</tr>
<tr>
<td>50μl/ml</td>
<td>5.05±0.01 (^e)</td>
</tr>
<tr>
<td>100μl/ml</td>
<td>5.59±0.09 (^d)</td>
</tr>
<tr>
<td>200μl/ml</td>
<td>5.75±0.12 (^c)</td>
</tr>
<tr>
<td>300μl/ml</td>
<td>5.99±0.16 (^b)</td>
</tr>
<tr>
<td>400μl/ml</td>
<td>6.35±0.22 (^a)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Days</th>
<th>Size of zone of inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 1</td>
<td>5.00±0.00 (^e)</td>
</tr>
<tr>
<td>Day 3</td>
<td>5.73±0.09 (^b)</td>
</tr>
<tr>
<td>Day 5</td>
<td>6.12±0.15 (^a)</td>
</tr>
<tr>
<td>Day 7</td>
<td>6.13±0.16 (^a)</td>
</tr>
</tbody>
</table>

**P-values**

<table>
<thead>
<tr>
<th>Source</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concentration</td>
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</tr>
<tr>
<td>Day</td>
<td>0.0001</td>
</tr>
<tr>
<td>Concentration*Day</td>
<td>0.0001</td>
</tr>
</tbody>
</table>

Values are expressed as means ± SEM for four replicates. Means within respect columns followed by similar small case letters are not significantly different at P <0.0001 by Tukey’s HSD test.

Figure 4.2 Interactive effects of concentration of *O. kilimandscharicum* oil with day on size of zones of inhibition.
4.4 Insecticidal activity of *O. kilimandscharicum* on *S. zeamais* in maize grains under laboratory conditions

4.4.1 Insecticidal property of *O. kilimandscharicum* oil on *S. zeamais*

Maize grains artificially infested with adult *S. zeamais* and treated with *O. kilimandscharicum* oil indicated insecticidal properties of *O. kilimandscharicum* oil. At 5μl concentration level of *O. kilimandscharicum* oil, mortality of *S. zeamais* was significantly (p<0.0001) lower than all other concentration levels (Table 4.3). There was however no mortality of *S. zeamais* in untreated maize grains. Mortality of *S. zeamais* at 105μl, 90μl, 60μl, 45μl, 30 μl and 5 μl concentration levels of *O. kilimandscharicum* oil was significantly higher (p<0.0001) than in untreated maize grains (Table 4.3). In addition, mortality of *S. zeamais* was significantly different (p<0.0001) in 105μl, 45μl, 30 μl and 5 μl concentration levels. However, mortality of *S. zeamais* was not significantly different at 60 μl and 90 μl concentration levels of *O. kilimandscharicum*.

Mortality was high on first day of exposure in maize treated with *O. kilimandscharicum* oil and reduced as days increased. Mortality of *S. zeamais* significantly differed (p<0.0001) in day 1, 3 and 5. Significant interactive effects (p<0.0001) were observed between concentrations and days on mortality of *S. zeamais* (Figure 4.3).
Figure 4.3: Interactive effects of concentration of *O. kilimandscharicum* oil with day on mortality of *S. zeamais*

Table 4.3: Mortality (mean ± S.E) of *S. zeamais* on day 1, 3, 5 and 7 after artificially infested maize samples were treated with different amounts of *O. kilimandscharicum* oil.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mortality of <em>S. zeamais</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>105μl</td>
<td>0.45±0.40&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>90μl</td>
<td>0.35±0.12&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>60μl</td>
<td>0.34±0.16&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>45μl</td>
<td>0.28±0.90&lt;sup&gt;bc&lt;/sup&gt;</td>
</tr>
<tr>
<td>30μl</td>
<td>0.14±0.50&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>15μl</td>
<td>0.09±0.40&lt;sup&gt;cd&lt;/sup&gt;</td>
</tr>
<tr>
<td>5μl</td>
<td>0.07±0.40&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>untreated maize</td>
<td>0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Days</th>
<th>Mortality of <em>S. zeamais</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 1</td>
<td>1.13±0.18&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Day 3</td>
<td>0.17±0.06&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Day 5</td>
<td>0.07±0.04&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Day 7</td>
<td>0</td>
</tr>
</tbody>
</table>

**P- values**

- Concentration: 0.0001
- Day: 0.0001
- Concentration*Day: 0.0001

Values are expressed means ± SEM for four replicates. Means within respect columns followed by similar small case letter(s) are not significantly different at P <0.0001 by Tukey’s HSD test.
4.5 Insecticidal activity of *O. kilimandscharicum* oil against *S. zeamais* in maize grains stored in granary.

4.5.1 Insecticidal property of *O. kilimandscharicum* on *S. zeamais* for six months

The on-farm experiments revealed that *O. kilimandscharicum* oil exhibited grain protection ability against *S. zeamais* infestations. At 10ml concentration level of *O. kilimandscharicum* oil, the numbers of holed grains were significantly lower (p<0.0001) than all other *O. kilimandscharicum* concentration levels (Table 4.4). In maize grains treated with Actellic Super™ the number of holed grains were significantly lower (p <0.0001) than maize grains treated with *O. kilimandscharicum* oil (Table 4.4). The number of holed grains in Actellic Super™ and all *O. kilimandscharicum* oil concentration levels were however significantly lower (p<0.0001) than in untreated maize grains. Significant interactive effects (p<0.0001) were observed between concentrations and number of months on the number of holed kernels (Figure 4.4).

Weight loss (in grams) revealed that as infestation of maize grains by *S. zeamais* increased, so did the weight loss. In 10ml concentration level of *O. kilimandscharicum* oil, weight loss (in grams) was significantly (p<0.0001) lower than all other *O. kilimandscharicum* oil doses (Table 4.4). Weight loss in maize grains was significantly higher (p<0.0001) in untreated maize grains than oil doses (Table 4.4). However, *O. kilimandscharicum* oil concentration levels differed significantly (p<0.0001). Weight loss in maize treated with Actellic super was significantly lower (p<0.0001) than the oil doses. Significant interactive effects (p<0.0001) were observed between concentrations and number of months on weight loss (Figure 4.5).
Table 4.4: Mean number of holed maize grains and weight loss in maize treated with different amounts of *O. kilimandscharicum* oil for six months storage.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Number of Holed kernels</th>
<th>Weight Loss</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated maize</td>
<td>2.27±0.08&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.35±0.14&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>2ml</td>
<td>1.65±0.18&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.17±0.14&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>4ml</td>
<td>1.47±0.18&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.02±0.14&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>6ml</td>
<td>0.92±0.19&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.66±0.14&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>8ml</td>
<td>0.73±0.18&lt;sup&gt;e&lt;/sup&gt;</td>
<td>0.58±0.12&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>10ml</td>
<td>0.73±0.16&lt;sup&gt;f&lt;/sup&gt;</td>
<td>0.46±0.11&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td>Actellic super</td>
<td>0.30±0.09&lt;sup&gt;g&lt;/sup&gt;</td>
<td>0.34±0.09&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Months</th>
<th>Number of Holed kernels</th>
<th>Weight Loss</th>
</tr>
</thead>
<tbody>
<tr>
<td>Month 1</td>
<td>0.21±0.10&lt;sup&gt;f&lt;/sup&gt;</td>
<td>0.15±0.07&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td>Month 2</td>
<td>0.51±0.14&lt;sup&gt;e&lt;/sup&gt;</td>
<td>0.33±0.10&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>Month 3</td>
<td>0.85±0.19&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.52±0.12&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Month 4</td>
<td>1.48±0.15&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.02±0.11&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Month 5</td>
<td>1.89±0.11&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.32±0.09&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Month 6</td>
<td>2.09±0.09&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.48±0.08&lt;sup&gt;a&lt;/sup&gt;</td>
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<table>
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<th>P- values</th>
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<td>Concentration</td>
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<td>Month</td>
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<tr>
<td>Concentration*Month</td>
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</table>

Values are expressed means ± SEM for four replicates. Means within respect columns followed by similar small case letters are not significantly different at P <0.0001 by Tukey’s HSD test.
Figure 4.4 Interactive effects of concentration of *O. kilimandscharicum* oil with month on number of holed kernels by *S. zeamais*.

Figure 4.5 Interactive effects of concentration of *O. kilimandscharicum* oil with month on weight loss of stored maize grains by infestation of *S. zeamais*. 
4.5.2 Replenishment of *O. kilimandscharicum* oil in maize grains at the end of third month

When *O. kilimandscharicum* oil was replenished in the fourth month at 10 ml, 8 ml and 6 ml the number of holed maize grains and weight loss reduced remarkably (Table 4.5). The number of holed maize grains were significantly higher (p<0.0001) in untreated maize grains than in all *O. kilimandscharicum* oil doses. However, the doses of *O. kilimandscharicum* oil differed significantly (p<0.0001) from one another (Table 4.5). Significant interactive effects (p<0.0001) were observed between concentration and month on the number of holed kernels (Figure 4.6) Similarly weight loss of stored maize grains after replenishing *O. kilimandscharicum* oil after the third month was significantly higher (p<0.0001) in untreated maize grains than all oil doses. Significant interactive effects (p<0.0001) were observed between concentration and month on weight loss of stored maize grains (Figure 4.7)
Table 4.5: Mean number of holed maize grains and weight loss in months on replenishing *O. kilimandscharicum* oil in maize after the third month.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Number of Holed kernels</th>
<th>Weight loss</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated maize</td>
<td>2.36±0.07&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.99±1.70&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>6ml</td>
<td>0.58±0.14&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.03±0.35&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>8ml</td>
<td>0.47±0.11&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.59±0.28&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>10ml</td>
<td>0.34±0.10&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.35±0.19&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Months</th>
<th>Number of Holed kernels</th>
<th>Weight loss</th>
</tr>
</thead>
<tbody>
<tr>
<td>Month 4</td>
<td>0.81±0.25&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.52±0.19&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Month 5</td>
<td>1.43±0.18&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.92±0.15&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Month 6</td>
<td>1.76±0.14&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.19±0.12&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

**P-values**

<table>
<thead>
<tr>
<th></th>
<th>Concentration</th>
<th>Month</th>
<th>Concentration*Month</th>
</tr>
</thead>
<tbody>
<tr>
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<td>0.0001</td>
<td></td>
</tr>
<tr>
<td>Month</td>
<td>0.0001</td>
<td>0.0001</td>
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</tr>
<tr>
<td>Concentration*Month</td>
<td>0.0001</td>
<td>0.0001</td>
<td></td>
</tr>
</tbody>
</table>

Values are expressed as means ± SEM for four replicates. Means within respect columns followed by similar small case letters are not significantly different at P <0.0001 by Tukey’s HSD test.

Figure 4.6 interactive effects of concentration of *O. kilimandscharicum* oil with month on number of holed kernels by *S. zeamais* on replenishing oil.
Figure 4.7 interactive effects of concentration of *O. kilimandscharicum* oil with month on weight loss of stored maize grains by infestation *S. zeamais* after replenishing oil.

### 4.5.3 Pre-treatment of baglets of maize grains with *A. indica* and *W. ugandensis* extracts and grains with *O. kilimandscharicum* oil

Results of pre-treating the baglets containing maize grains treated with *O. kilimandscharicum* oil with aqueous extract of *A. indica* and *W. ugandensis* showed improved protection of maize grains against *S. zeamais*. The number of holed maize grains was significantly higher (p<0.0001) in untreated maize grains stored in unpretreated baglets than in treated maize grains stored in pre-treated baglets (Table 4.6). There were no significant differences in the number of holed kernels in baglets pre-treated with aqueous extracts (Table 4.6). Significant interactive effect (p<0.0001) was observed between treatment and month on the number of holed kernels (Figure 4.8).
Weight in (grams) loss in maize treated with *O. kilimandscharicum* oil and stored in baglets pre-treated with aqueous extracts of *A. indica* and *W. ugandensis* was significantly (*p*<0.0001) lower than in untreated maize grains stored in unpre-treated baglets. There was no significant difference in weight loss in baglets treated with aqueous extracts of *W. ugandensis* and *A. indica*. Significant interactive effect (*p*<0.0001) were observed between treatments and month on weight loss of stored maize grains (Figure 4.9).

![Figure 4.8 Interactive effects of maize treated with *O. kilimandscharicum* oil, stored in baglets treated the aqueous extracts of *A. indica* and *W. ugandensis* with month on number of holed kernels by *S. zeamais*](image-url)
Table 4.6: Mean number of holed maize grains and weight loss in maize treated with *O. kilimandscharicum* oil and stored in baglets treated the aqueous extracts of *A. indica* and *W. ugandensis* for six months.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Number of Holed kernels</th>
<th>Weight loss</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated maize</td>
<td>2.19±0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.57±0.07&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>W. ugandensis</em> + oil</td>
<td>0.91±0.19&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.60±0.13&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>A. indica</em> + oil</td>
<td>0.89±0.19&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.59±0.12&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Months</th>
<th>Number of Holed kernels</th>
<th>Weight loss</th>
</tr>
</thead>
<tbody>
<tr>
<td>Month 1</td>
<td>0.51±0.22&lt;sup&gt;f&lt;/sup&gt;</td>
<td>0.31±0.13&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td>Month 2</td>
<td>0.66±0.28&lt;sup&gt;e&lt;/sup&gt;</td>
<td>0.48±0.21&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Month 3</td>
<td>0.73±0.31&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.52±0.22&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Month 4</td>
<td>1.76±0.13&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.15±0.12&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Month 5</td>
<td>2.07±0.09&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.43±0.09&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Month 6</td>
<td>2.27±0.07&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.61±0.06&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

**P- values**

<table>
<thead>
<tr>
<th></th>
<th>Concentration</th>
<th>Month</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.0001</td>
<td>0.0001</td>
</tr>
<tr>
<td>Treatment*Month</td>
<td>0.0001</td>
<td>0.0001</td>
</tr>
</tbody>
</table>

Values are expressed as means ± SEM for four replicates. Means within respect columns followed by similar small case letters are not significantly different at P<0.0001 by Tukey’s HSD test.
Figure 4.9 Interactive effects of maize treated with *O. kilimandscharicum* oil, stored in baglets treated the aqueous extracts of *A. indica* and *W. ugandensis* with month on weight loss.
CHAPTER FIVE

DISCUSSION

5.1 Effect of Ocimum kilimandscharicum oil against Aspergillus flavus

Antifungal test of essential oils extracted from *O. kilimandscharicum* showed that the oil exhibit antifungal activity against *A. flavus*. There was total inhibition on the growth of the fungus at 26μl/ml, 20μl/ml, 13.33μl/ml and 6.66μl/ml concentration levels using agar disc diffusion method. However, there was slight growth in 3.33 μl/ml and rapid growth in plain PDA. It was revealed in this study that, increase in the antifungal activity of the extracts was enhanced by increase in the concentration level of the extracts. This finding agrees with the report of Mares *et al.* (2004); Sharma and Tripath, (2007) who established that higher concentration of antimicrobial substance showed increase in growth inhibition.

Results obtained in this study observed that disc diffusion method was almost similar to agar disc diffusion method. The highest concentration level of 400μl/ml had the largest zone of inhibition while lowest concentration level of 50 μl/ml had the least zone of inhibition. No inhibition was observed in plain PDA which was set as a control treatment. The size of zone of inhibition depended on the concentration of the essential oil as observed in agar disc diffusion method. Compounds such as 1, 8-cineole, camphene, 4-terpeneol, limonene, α-terpineol, α-terpineol, endo-borneol, endo-borneol, transcaryophyllene, camphor, myrtenol, and linalool are contained in aqueous extract of leaves of *O. kilimandscharicum* (Eliningaya *et al.* (2009). Leaves also contain triterpenoids saponins, tannins, sterols, flavonoids, carbohydrates and proteins (Paschapur *et al.*, 2009). These chemical constituents are principle behind
various biological activities. Sethi et al. (2012) reported that camphor is the major component in *O. kilimandscharicum* oil while Gupta and Saxena (2010) documented that the oil possesses good to moderate antifungal activity.

It has been documented that essential oils with anti-fungal effectiveness is able to constantly cause morphological changes in *Aspergillus* species. These are distortion of hyphae, aberrant development of conidiophores, failure of sporulation and loss of pigmentation. (Rasooli and Abyaneh, 2004). Results of the present work showed that *O. kilimandscharicum* oil inhibits growth of the fungi as indicated by growth inhibition. The inhibitory effect of the tested extracts might be due to natural bioactive materials present in these extracts (Rasooli and Abyaneh, 2004).

Natural compounds such as diterpenes, monoterpenes and hydrocarbons with various functional groups have been found in essential oils. Many other researchers have since reported on antimicrobial (Shittu et al., 2002; Benkeblia, 2004; Burt, 2004) and antifungal activities of essential oils in medicinal research, food applications and other areas. However, little has been published previously on the use of essential oils as antimold agents on food/feed products (Shittu et al., 2002; Burt, 2004). This study shows the essential oils from *O. kilimandscharicum* can be used as antifungal against *A. flavus*.

**5.2 Insecticidal activity of *O. kilimandscharicum* on artificially infested maize grains**

Results from this study revealed that the oil has contact toxicity property since mortality was high after 24 hour exposure. There was 100% mortality of *S. zeamais* at 105μl/ml concentration level of *O. kilimandscharicum* and 0% mortality at 5 μl/ml
concentration level after 24 hour exposure. Mortality of S. zeamais was also 0% on untreated maize grains. Mortality of S. zeamais was high on the first day of exposure in maize treated with O. kilimandscharicum at 105 μl, 90μl, 60μl, 45μl and 30μl doses and reduced as days increased. High mortality in the first day of exposure might be due to contact toxicity of its major constituent camphor.

This study agrees with Obeng-Ofori et al. (1998) who documented that O. kilimandscharicum at 100mg/filter paper and 100μg/insect caused over 93% and 100% mortalities in Sitophilus granarius, S. zeamais and Prostephanus truncatus. However, only 70% and 100% mortality in Tribolium castaneum was observed after 24 hour exposure. According to Jembere and Hassanali (2001) dried ground leaves and essential oil of O. kilimandscharicum in doses of 25.0g leaves and 0.3g essential oil per 250g grain (sorghum or maize) killed 100% of Sitotroga cerea and Rhyzopertha dominica and Sitophilus zeamais in 2 days. The best repellent activity was seen by 0.3 g essential oil/250 g grains against Sitophilus zeamais.

Toxic effects of plant extracts in the Institute for stored Product Protection were initially done by Obeng-Ofori et al. (1998) on extract of the medicinal plants of O. kilimandscharicum, O. suave and O. keneyense. Depending on the Ocimum species, eugenol, 1, 8 cineol and camphor has been identified as main compounds of the essential oils. The compounds were found to greatly affect beetles of the species Tribolium castaneum, Lasioderma serricorne, Prostephanus truncatus, Sitophilus granarius and S. zeamais when mixed to grain. Complete control was achieved after 24 hr at a dosage of 0.5 μl/kg or 0.5 mg/kg of grain. Furthermore, it was established that admixture of these compounds with low quantities of vegetable oils like
sunflower seed oil or sesame oil increased toxicity to insects and persistency (Obeng-Ofori et al., 1998).

5.3 Long term insecticidal activity of *O. kilimandscharicum* on *S. zeamais*

On farm studies revealed that *O. kilimandscharicum* oil was effective against *S. zeamais*. However, insecticidal activity of the oil varied with dose used. At 10ml, 8ml and 6ml doses of *O. kilimandscharicum* efficacy against *S. zeamais* lasted for three months. At 4ml and 2ml doses of *O. kilimandscharicum* efficacy against *S. zeamais* lasted for one month after treatment. However, in 10ml, 8ml, 6ml, 4ml, and 2ml doses of *O. kilimandscharicum* oil the number of holed grains differed significantly \((p \leq 0.05)\) from control treatment. The percentage weight loss in maize was proportional to the number of holed grains. The weight loss was below 50% in 10ml, 8ml and 6ml doses of *O. kilimandscharicum* after six months of storage. In 4ml and 2ml doses of *O. kilimandscharicum*, percentage weight loss in maize grains was above 50% after six months of storage. Percentage weight loss in untreated maize grain (control treatment) differed significantly \((p \leq 0.05)\) with every *O. kilimandscharicum* dosage.

When *O. kilimandscharicum* oil was replenished after the third month of storage, the number of holed maize grains significantly \((p \leq 0.05)\) reduced in 10ml, 8ml and 6ml doses of *O. kilimandscharicum*. In addition efficacy of the oil against *S. zeamais* improved with an extra month. Percentage weight loss was also proportional to the number of holed grains. The weight loss was below 20% in 10ml, 8ml, and 6ml doses of *O. kilimandscharicum*. Every *O. kilimandscharicum* dose replenished after the third month differed significantly \((p \leq 0.05)\) with untreated maize grains.
According to Ileke and Oni (2011); Mbailao et al., (2006); extracts from Lamiaceae Family are effective in causing mortality of weevils may be as a result of active compounds such as flavanoids, alkaloids, steroids, glycosides and terpenoids against weevils. Furthermore, compounds such as saponins, glycosides, flavanoids and phenols have toxic effects on Coleopterans (Ekeh et al., 2013). It’s also possible that some of the extracts came into contact with insects’ spiracle to further mortality.

Death of some weevils might have been due to starvation (Kemabonta and Falodu, 2013) because coating the grains with the extracts minimized contact between the grains and the weevils. Compounds found in Lamiaceae such as monomeric flavanoids, glycosides, terpenoids and tannins acted as feeding deterrent (Shadia et al., 2007; Ekeh et al., 2013) and thus enhanced mortality by starvation.

5.4 Pretreatment of baglets containing treated maize grains with aqueous extracts of A. indica and W. ugandensis

Pretreatment of baglets with aqueous extract of A. indica and W. ugandensis improved the efficacy of the O. kilimandscharicum oil against S. zeamais. The numbers of holed grains were lower in treated maize stored in pretreated baglets than in untreated maize stored in unpretreated baglets after six months of storage. In addition significant difference (p≤0.05) was observed in the number of holed grains in treated maize grains stored in pretreated baglet and treated maize in unpretreated baglet. Percentage weight loss was also proportional to the number of holed grains. Weight loss below 30% in pretreated baglets and below 40% in treated maize grains stored in unpretreated baglet after six months of storage. Percentage weight loss in untreated maize grains in unpretreated baglets was above 50%. Reduced weight loss in
pretreated baglets could as a result of active ingredients present in the three plant extracts.

This study agrees with Adhikary (1981) who found that neem leaf treatment of corn stored in sacks or unpeeled corn cobs held in bins was quite simple and effective against S. zeamais, Tribolium spp., Rhyzopertha dominica, and Cathartus spp. Muda (1984), documented mixing neem leaves with paddy grain in a proportion of 2 to 100 parts(wt/wt), bag treatment with neem leaf extract, or placing barriers of neem leaves between bags and storage floor, significantly reduced the infestation by S. oryzae and R. dominica to paddy grain stored in 40 kg jute bags for 3 months.

Azadirachtin is a compound derived from A. indica tree (Isman 2006). Azadirachtin is documented to be active against stored product pests acting as repellent, sterilant, insect growth regulator, feeding deterrent and as anti-ovipositional. Neem extracts eliminates insect pests with soft cuticle or their juveniles, although it is basically anti-ovipositional, insect growth regulator, feeding deterrent and repellent (Jacobson, 1988).

According to Kubo (1995), methanolic extract of W. ugandensis was found to be potential insect antifeedant activity against Spodoptera Exempta. Based on fractionation guided by the assay, three antifeedants were isolated from the fruits, leaves and bark of W. ugandensis. Feeding of larvae of two species of Africam armyworm, the monophagous Spodoptera exempta and the polyphagous Spodoptera littoralis was inhibited by Muzigadial and warburganal at 0.1ppm in a regular leaf disk method (Kubo and Nakanishi, 1977).
Kubo et al. (1977) documented that ugandensial and polygadial being a potent antifeedant for Spodoptera but less active. Drimane sesquiterpenoids are also shown to have insect antifeedant property (Frum, 2006). According to were et al. (2010), stem bark of *W. ugandensis* exhibit antiplasmodial activity against *Plamodium knowlesi* and *Plamodium berghei*. Warburgia species possess high pharmaceutical value, both for humans and livestock. This is due the abundance of drimane and colorotane sesquiterpenoids (Frum *et al.*, 2005).

Opiyo et al. (2015) documented that essential oil of *W. ugandensis* was effective against *S. zeamais* and exhibited 100% mortality after 21 days. The extracts were effective as actellic dust and completely inhibited emergence of adults’ insects. Polygadial, ugandensolide and warburganal were best in growth inhibition (Opiyo *et al.*, 2015).
CHAPTER SIX
CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

From this study it can be concluded that:

1. *O. kilimandscharicum* oil is effective as antifungal and more specifically being active against *A. flavus*. The activity of *O. kilimandscharicum* oil was concentration-dependent in that higher concentration was more active than low concentration with respect antifungal activity. Concentration level above 100μl/ml of *O. kilimandscharicum* inhibited totally growth *A. flavus* in petri dishes.

2. Essential oil of *O. kilimandscharicum* has insecticidal property and more specifically being active against *S. zeamais*. The activity was also concentration dependent totally. In artificially infested maize grains, mortality of *S. zeamais* was high on the first day of exposure to the essential and this probably demonstrates the contact toxicity of essential oil.

3. Long-term insecticidal activity of essential oil of *O. kilimandscharicum* shows that the oil can protect maize against infestation of *S. zeamais* in granary for three months. The activity was concentration dependent where 10ml per 1Kg of maize was selected as the best dosage. Replenishing essential oil on maize grains after 3 months of storage increased its efficacy.

4. Pre-treatment of baglets with aqueous extracts of *A. indica* and *W. ugandensis* is useful as it enhances protection of stored threshed maize grains against *S. zeamais*. 
Pre-treatment has therefore an added advantage to treating maize with *O. kilimandscharicum* oil.

### 6.2 Recommendations

This study recommends that:

1. Essential oil of *O. kilimandscharicum* to be used for postharvest protection maize against infestation by *S. zeamais*.
2. Appropriate technology should be sought to develop cheap apparatus for the extraction of essential oil at farm level
3. Extraction, processing, packaging and marketing of essential oil of *O. kilimandscharicum* to done for use in the management of post harvest pest and fungi on maize.
4. Further experiments are needed to verify the persistence of oil toxicity on eggs and larvae of *S. zeamais* before potential applications or possible formulations.
5. Studies are carried out on what happens if 3 months replenishment of *O. kilimandscharicum* oil on maize is continued beyond 6 months.
6. Further studies are carried out to establish whether the same plant extracts give same protection to stored products against other post harvest fungi and pest
REFERENCES


Project, Egerton University/ Michigan State University with the support by the United States Agency for International Development/Kenya.


