ANTIFUNGAL ACTIVITIES OF ESSENTIAL PLANT OILS ON MYCOTOXIGENIC MOULDS ISOLATED FROM MAIZE IN SELECTED AREAS OF WESTERN KENYA

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DECLARATION AND RECOMMENDATION

Declaration

This thesis is my original work and has not been presented in part or as a whole for examination in any other University.

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Recommendation

This thesis has been submitted to the Graduate School with our approval as University supervisors.

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DEDICATION

This work is dedicated to my husband Eliud Okumu Ongowo,

My son Trevor Adams,

My Mum, Dad, Brothers and Sisters.

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ABSTRACT

Moulds destroy more than 30% of crop yields in developing countries and produce potentially poisonous mycotoxins. The most prevalent moulds on foods are *Aspergillus, Fusarium, Penicillium, Rhizopus* and *Mucor*. Kenya has experienced dramatic outbreaks of mycotoxin poisoning resulting in loss of lives. Existing strategies for control of moulds mainly based on moisture reduction have been ineffective in the warm and humid areas and methods like solar driers are expensive for the small scale farmers. The aim of this study was, to determine the antifungal activity of essential oils on moulds isolated from maize growing in selected areas in Western Kenya. Moulds were isolated from 30 samples of good and mouldy maize and collected from three zones in Western Kenya (Trans-nzoia, Kakamega and Kuria districts). They were identified using cultural and morphological characteristics and their quantification done by determining microbial load. Mycotoxin extracts from the maize samples were spotted on TLC plates for detection of aflatoxins B_1 (AFB_1) and G_1 (AFG_1). Five essential oils were extracted from selected aromatic plants (*Tarchonanthus camphoratus, Artemisia vulgare, Piper capense, Foeniculium vulgare* and *Rosmarinus officinalis)* using hydro-distillation and screened for their antifungal activity against mycotoxigenic moulds to determine the most active oil.

Two main genera (*Aspergillus* and *Fusarium*) were isolated and identified. Among the genus *Aspergillus,* twelve mycotoxigenic species and two atoxigenic species were identified and among the genus *Fusarium,* fourteen mycotoxigenic species were identified. In all the three districts, the most frequent *Aspergillus* and *Fusarium* species on maize were *A. flavus* at 23.1% and *F. proliferatum* at 20.3% frequency. Out of the samples analyzed, 84.6% were positive for the aflatoxins and 15.4% did not have any detectable amount of the toxins. The total percentage of the samples that tested positive for the AFB_1 was 83.8%, 9.7% had AFG_1 and 6.5% had both the AFB_1 and AFG_1 toxins. After the screening, the most bioactive essential oil that induced large inhibition zones was found to be *T. camphoratus*. The oil was active against all the twelve *Aspergillus* and fourteen *Fusarium* species. The oil had minimum inhibitory concentration (MIC) values ranging from 30 mg/ml to 470 mg/ml. These results show that the essential oil of *T. camphoratus* has antifungal activities against *Aspergillus* and *Fusarium* species that are the producers of poisonous mycotoxins found in maize. This oil can be used in food preservation systems to inhibit the growth of moulds and retard subsequent mycotoxin production.

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LIST OF ABBREVIATIONS

AFB_1	Aflatoxin B_1
AFG_1	Aflatoxin G_1
DMSO	Dimethyl sulfoxide
FEMA	Federal Emergency Management Agency
MEA	Malt Extract Agar
MIC	Minimum Inhibitory Concentration
NIOSH	National Institute for Occupational Safety and Health
OTA	Ochratoxin A
OTB	Ochratoxin B
OTC	Ochratoxin C
PDA	Potato Dextrose Agar
STD	Standards
TLC	Thin Layer Chromatography

CHAPTER ONE
INTRODUCTION

1.1 Background information

Moulds are opportunistic biological agents of ubiquitous nature (Ryan and Ray, 2004). Because of their powerful arsenal of hydrolytic enzymes, these microorganisms can cause a high degree of deterioration when present in foods and are responsible for considerable economic losses (Souza *et al.*, 2005). Furthermore, they can act as potential producers of toxic metabolites, named mycotoxins, which are potentially poisonous to consumers' health. The most common moulds that develop on foods and feeds include those from the genera *Aspergillus, Penicillium, Fusarium, Rhizopus* and *Mucor*. Moulds cause extensive damage on foods, feeds and other agricultural commodities in the field, during transportation, storage and processing, leading to postharvest losses. They are known to destroy 10 to 30% of the total yield of crops and more than 30% of perishable crops in developing countries by reducing their quality and/or quantity (Fandohan *et al.*, 2003). They also lower the nutritional and sale value of the produce (Agrios, 1997). One of the most destructive activities of moulds in foodstuffs occurs in stored seeds and grains (Kwun-chung and Bennet, 1992). Many other moulds infect agricultural commodities or contaminate food produce with several mycotoxins. Most of the deterioration of grains and legumes after harvest is caused by several species of *Aspergillus* which are responsible for many cases of food and feed contamination (Abarca *et al.*, 1994; Katta *et al.*, 1995; Agrios. 1997).

Mycotoxins contaminate 25% of agricultural crops worldwide and are a source of morbidity and mortality throughout Africa, Asia and Latin America (Smith *et al.*, 1994). Mycotoxins are chemicals produced by fungi that are harmful to humans and domestic animals. These chemicals may contaminate staple foods and feeds worldwide, posing a number of significant food safety concerns (Schmale and Munkvold, 2009). They cause diseases referred to as mycotoxicoses in humans and animals (Agrios, 1997). Most mycotoxicoses are caused by the common and widespread moulds namely *Aspergillus, Penicillium* and *Fusarium.* They cause acute liver damage, induction of tumors and attack on the central nervous system, skin disorders and hormonal effects (Agrios, 1997; Ibrahim *et al.*, 2000; Oguz *et al.*, 2003). *Aspergillus* and *Penicillium* produce their toxins mostly in stored seeds and hay but also on commercially processed foods and feeds including meats, cheese, spices etc. Some of the toxins such as ochratoxins can persist in meat of animals fed on contaminated feed and can be transmitted to humans through the food chain. *Fusarium* produces its toxins primarily on maize or other grains infected in the field or in storage (Agrios, 1997). The most important mycotoxins are aflatoxins, deoxynivalenol, fumonisins, ochratoxins and zearalenones. Among these the most famous are the aflatoxins which are produced by *A. flavus, A. parasiticus* and several other species of *Aspergillus* in a wide variety of agricultural commodities including grains, legumes and nuts (Patten, 1981). Acute aflatoxicosis epidemics occur in several parts of Africa and Asia leading to death of several hundred people (Varga *et al.*, 2009). Aflatoxins are known to be potent hepatocarcinogens in animals and humans (Dvorackova, 1990). Ochratoxin A, which has been experimentally shown to be teratogenic, potent renal carcinogenic and immunosuppressive, is largely produced by *Aspergillus ochraceus* and less frequently by *A. niger* (Nielsen *et al.*, 2009). Kenya has experienced dramatic outbreaks of mycotoxin poisoning resulting in loss of lives. In 2004, an acute aflatoxicosis outbreak occurred in Machakos resulting in 125 deaths (Azziz -Baumgartner *et al.*, 2005), while cases of liver cancer in Uganda have been linked to high levels of aflatoxins in the country's food (Kaaya *et al.*, 2006).

The wide and indiscriminate use of chemical preservatives has been the cause of the appearance of resistant micro-organisms, leading to occurrence of emerging food borne diseases (Gibbons, 1992; Kaur and Arora, 1999; Akinpelu, 2001). Due to this, there is an increasing interest to obtain alternative antimicrobial agents to use in food conservation systems. It is well established that some plants contain compounds able to inhibit microbial growth (Naqui *et al.,* 1994; Matasyoh *et al.*, 2007, 2009). These compounds can have different structures and different action when compared with conventional antimicrobials used to control microbes (Nascimento *et al.*, 2000). The potential antimicrobial properties of plants had been related to their ability to synthesize several chemical compounds of relatively complex structures with antimicrobial activity (Nychas, 1996). Studies have shown that plant derivatives such as essential oils possess inhibitory activity against moulds (Souza *et al.*, 2005). The antimicrobial activities of essential oils are well recognized for many years (Cosentino *et al.*, 1999; Matasyoh *et al.*, 2007). This activity could act as chemical defense against plant pathogenic diseases. For example, higher plants have traditionally been used in folk medicine as well as to extend the shelf life of foods, showing inhibition against bacteria, fungi and yeast (Alves *et al.,* 2000; Sartoratto *et al.*, 2004). Most of their properties are due to essential oils produced by their secondary metabolism.

Use of essential oils with antimould activities in food preservation would provide a technology to ensure that the foods and feeds are free of moulds and mycotoxins. Such essential oils of recognized antimicrobial spectrum could appear in food preservation systems as main antimicrobial compounds or as adjuvant to improve the action of other antimicrobial compounds (Kaur and Arora, 1999). In addition to characterizing moulds associated with maize, this project aimed at identifying aromatic plants with essential oils that can control the growth of moulds in maize and, therefore inhibit the production of associated mycotoxins in the grains. These antimould essential oils can be used by farmers, food processors and consumers as cheap, affordable and environmentally-friendly antimould products for food preservation systems. This would help alleviate great losses of grains encountered due to moulds and prevent mycotoxin poisoning outbreaks.

1.2 Statement of the Problem

The ubiquitous nature of moulds, their ability to colonize diverse substrates and lack of effective control measures has contributed to the high incidences of mould and mycotoxin contamination in foods and feeds. Moulds destroy more than 30% of crop yields and mycotoxins contaminate 25% of agricultural crops worldwide. Acute mycotoxicoses epidemics occur in Africa leading to death of several hundred people. In 2004, an acute aflatoxicosis outbreak occurred in Kenya resulting in 317 cases and 125 deaths, while cases of esophageal cancer have been linked to high levels of fumonisins in Lake Victoria Basin (LVB). One of the available methods of controlling moulds is the use of synthetic chemical preservatives like sodium nitrate which have been the cause of appearance of resistant micro-organisms, leading to occurrences of emerging food borne diseases. Furthermore, other methods such as use of solar driers are expensive for the small scale farmers.

1.3 Objectives

1.3.1 General Objective

To control moulds and associated mycotoxins in maize using essential oils from aromatic plants.

1.3.2 Specific Objectives

1. To isolate, identify and characterize moulds associated with maize from Trans-nzoia, Kakamega and Kuria districts.
2. To quantify moulds associated with maize from the above selected areas.
3. To analyze maize samples from Trans-nzoia, Kakamega and Kuria districts for aflatoxins.
4. To screen essential oils from aromatic plants; *Artemisia vulgare, Piper capense, Foeniculium vulgare, Tarchonanthus camphoratus,* and *Rosmarinus officinalis* for antimould activities against the most common and damaging mycotoxigenic moulds associated with maize in order to select the most bio-active essential oil.
5. To conduct a detailed antimould analysis of the most bioactive essential oil.

1.4 Hypotheses

1. No moulds are found in maize collected from Trans-nzoia, Kakamega and Kuria districts.
2. Maize from Trans-nzoia, Kakamega and Kuria districts do not have the same quantity of moulds.
3. Maize samples from Trans-nzoia, Kakamega and Kuria districts do not contain aflatoxins.
4. Essential oils from selected aromatic plants have no antimould activities on mycotoxigenic moulds.
5. The most bioactive essential oil has no antimould activity against the mycotoxigenic mould species.

1.5 Justification

To design strategies for the reduction or elimination of mycotoxins in food and feed, knowledge about their fungal sources is needed, hence the need to identify and quantify the mycotoxin producing moulds in grains. The prevention of mycotoxin production in grains is an urgent task. To avoid the loss of lives due to acute mycotoxicoses outbreaks, there is need to improve food and health security through postharvest food protection. There is an increasing interest to obtain alternative antimicrobial agents from plant based sources in order to control the high incidences of moulds and mycotoxin contamination in foods and feeds. One way of achieving this is by screening and evaluating antimicrobial activities of bio-active essential oils from target aromatic plants occurring in the region. Existing strategies for control of moulds mainly based on moisture reduction have been ineffective in the warm and humid regions. Furthermore, methods like solar driers and chemicals are very expensive for the small scale farmers and also lead to resistance. Use of essential oils in food preservation would provide an environmentally friendly and affordable technology that ensures foods are free of moulds and mycotoxins. Aromatic plants have been used traditionally by the local people and their antifungal activities have been reported in literature. The bio-active oils that are environmentally friendly will improve food and health security through postharvest food protection.

CHAPTER TWO
LITERATURE REVIEW

2.1 Biology of moulds

Moulds include all species of microscopic fungi that grow in the form of multicellular filaments called hyphae. A connected network of these tubular branching hyphae has multiple, genetically identical nuclei and is considered a single organism, referred to as a colony or in more technical terms a mycelium. Moulds do not form a specific taxonomic or phylogenetic grouping, but can be found in the divisions *Zygomycota*, *Deuteromycota* and *Ascomycota*. Although some moulds cause disease or food spoilage, others are useful for their role in biodegradation or in the production of various foods, beverages, antibiotics and enzymes (Madigan and Martinko, 2005).

According to Ryan and Ray (2004), moulds derive energy from the organic matter in which they live. Typically, moulds secrete hydrolytic enzymes, mainly from the hyphal tips. These enzymes degrade complex biopolymers such as starch, cellulose and lignin into simpler substances which can be absorbed by the hyphae. Many moulds also secrete mycotoxins which, together with hydrolytic enzymes, inhibit the growth of competing microorganisms. Although moulds grow on dead organic matter everywhere in nature, their presence is only visible to the unaided eye when mould colonies form. Most fungi are aerobic (use oxygen) and are found almost everywhere in varying quantities. They consume organic matter wherever humidity and temperature are sufficient. In artificial environments like buildings, humidity and temperature are often stable enough to foster the growth of mould colonies, commonly seen as a downy or furry coating growing on food or other surfaces (NIOSH, 2008). Some moulds can begin growing at temperatures as low as 2°C. When conditions do not enable growth, moulds may remain alive in a dormant state depending on the species, within a large range of temperatures before they die (Ryan and Ray, 2004).

Moulds cause loss of millions of dollars to the economy every year and, even worse, may be a menace to human and animal health. Toxin-producing fungi may invade at pre-harvesting period, harvest-time, during post-harvest handling and in storage. Toxigenic fungi can be divided into three groups: field fungi namely, genus *Fusarium*, for example *F. moniliforme*, *F. roseus*, *F. trincinctum* and *F. nivale*; Storage fungi which include the genus *Aspergillus* and *Penicilium*, for example *A.*

flavus, and *A. parasiticus*; and advanced deterioration fungi which normally do not infect intact grains but easily attack damaged ones and require high moisture content. Examples of the third group are *A. clavatus, A. fumigatus, Chaetomium, Scopulariopsis, Rhizopus, Mucor,* and *Absidia* (Makun *et al.,* 2009). Postharvest diseases caused by moulds destroy 10 to 30% of the total yields of crops and in some perishable crops, especially in developing countries they destroy more than 30% of the crop yields (Fandohan *et al*., 2003). Moulds not only contaminate air but also our food. As they grow on food, they produce enzymes that break down the food resulting to spoilage (Kung'u, 2005).

Moulds are ubiquitous in nature, and mould spores are a common component of household and workplace dust (NIOSH, 2008). However, when mould spores are present in large quantities, they can present a health hazard to humans, potentially causing allergic reactions and respiratory problems. Some moulds, called toxigenic moulds, also produce by-product mycotoxins, which in high doses can be detrimental to human and animal health (FEMA, 2005). Exposure to high levels of mycotoxins can lead to neurological problems and in some cases death. Prolonged exposure for example daily workplace exposure can be particularly harmful. The most common moulds are *Aspergillus, Fusarium, Penicillium, Mucor, Acremonium, Cladosporium* and *Rhizopus* (Ryan and Ray, 2004).

2.2 Factors affecting production of mycotoxins in grains and their effects

Mycotoxins are toxic secondary metabolites of fungal origin and contaminate agricultural commodities before or under post harvest conditions (Bennet and Klich, 2003). They are mainly produced by fungi in the *Aspergillus, Fusarium* and *Penicillium* genera. When ingested, inhaled or absorbed through the skin, mycotoxins cause lowered performance, sickness or death in humans and animals. Factors that contribute to mycotoxin contamination in Africa include environmental, socio-economic and food production. Environmental conditions especially high humidity and temperature favors fungal proliferation resulting to contamination of food and feeds. The resulting implications include immunosuppression, impaired growth, various cancers and death depending on the type, period or amount of exposure (Wagacha and Muthomi, 2008).

The production of toxins is dependant on the surrounding environments but the toxins vary greatly in their effects depending on the organism infected, its susceptibility, metabolism, and defense mechanisms (Mazid *et al.*, 2011). Some of the health effects found in animals include death, identifiable diseases or health problems, weakened immune systems without specificity to a toxin, and as allergens or irritants (Bennet and Klich, 2003). Mycotoxins can appear in the food chain as a result of fungal infection of crops, either by being eaten directly by humans, or by being used as livestock feed. They greatly resist decomposition or being broken down in digestion, so they remain in the food chain in meat and dairy products. Even temperature treatments, such as cooking and freezing, do not destroy mycotoxins. The growth of fungi in crops is the main cause of toxin formation and is related to the concentration of the toxic substances.

Many factors are involved in enhancing the formation of mycotoxins. They include plant susceptibility to fungal infestation, moisture content and physical damage of seeds due to pests. Mycotoxins contaminate 25% of agricultural commodities worldwide and are a source of mortality throughout the world. Consumption of mycotoxin-contaminated foods has been associated with several cases of human poisoning or mycotoxicoses (Munimbazi and Bullerman, 1996). Currently, over 200 mycotoxins are known but only those occurring naturally in foods i.e. aflatoxins, ochratoxin, citrinin, patulin, ergot alkaloids, fumonisins, zearalenones and trichothecenes are of significance in terms of food safety. These are produced by species of *Aspergillus, Penicillium*, and *Fusarium* (Moturi, 2008). Mycotoxins differ in their chemical formula, in their products and conditions under which they are produced, in their effects on various animals and humans, and in their degree of toxicity (Agrios, 1997). The most important ones in terms of economic importance, incidence and toxicity in agricultural produce are aflatoxins, deoxynivalenol, fumonisins, ochratoxins and zearalenones produced by *A. flavus, A. parasiticus*, *F. moniliforme, A. ochraceus* and *F. graminearum*, respectively (FAO, 1999). Most important of these are the aflatoxins, which are toxic to humans and domestic animals. They are produced in infected cereal seeds, most legumes especially groundnuts, cotton seed, fishmeal, nuts etc. Aflatoxins exist in a variety of derivatives with varying effects. Some of the toxins for example, ochratoxins can persist in meat or milk of animals fed on contaminated feed and can be transmitted to humans through the food chain (Lanyasunya *et al.*, 2005). Possible intervention strategies include good agricultural practices like early harvesting, proper drying, sanitation, proper storage and insect management. Other possible interventions include biological control, decontamination or breeding for resistance as well as surveillance and awareness creation. There is need for efficient, cost effective sampling and analytical methods that can be used for detection analysis of mycotoxins (Wagacha and Muthomi, 2008).

2.3 Major groups of mycotoxins produced by moulds

Aflatoxins are a group of mycotoxins produced by some *Aspergillus* species like *A. flavus* or *A. parasiticus* (Schmale and Munkvold, 2009). Aflatoxins are of various types, namely aflatoxin B1, B2, G1, G2 and the hydroxylated metabolite M1 with aflatoxin B1 as the most frequently occurring (Martins *et al.*, 2001). Aflatoxins can be found on a wide range of commodities including cereals, nuts, spices, figs and dried fruit. Aflatoxin M1, the metabolite of Aflatoxin B1, is found in milk and dairy products. Aflatoxin B1 is one of the most potent hepato-carcinogens known and hence levels of aflatoxins in the diet are an important consideration for human health (Yin *et al.*, 2008).

Ochratoxin is a mycotoxin that comes in three secondary metabolite forms; A, B, and C. All are produced by *Penicillium* or *Aspergillus* species, like *A. ochraceus* or *P. viridicatum*, with ochratoxin A as the most prevalent/relevant fungal toxin of this group (Wikipedia, 2010). The three forms differ in that ochratoxin B (OTB) is a nonchlorinated form of ochratoxin A (OTA) and that ochratoxin C (OTC) is an ethyl ester form of ochratoxin A (Bayman and Baker, 2006). *Aspergillus ochraceus* is found as a contaminant of a wide range of commodities like cereals, coffee, dried fruits and red wine. *Aspergillus carbonarius* is the main species found on grapes that releases toxins during the juice making process (Mateo *et al.*, 2007). Ochratoxin A has been labeled as a carcinogen, nephrotoxin and has been linked to tumors in the human urinary track (Abarca *et al.*, 1994).

Fumonisins are a group of mycotoxins produced by *Fusarium* species like *F. moniliforme* and *F. proliferatum.* Maize is the mainly affected commodity. Conditions favoring fumonisins formation are drought stress followed by warm, wet weather. Fumonisins have various effects on animals like causing pulmonary edema in swine. They are also suspected to influence the formation of esophageal cancer in humans (Schaafsma and Hooker, 2007).

Zearalenone is a mycotoxin produced by *Fusarium* species like *F. graminearum*. Zearalenone contamination is economically important in maize and hay. High humidity and low temperature favours the production of this toxin by *F. graminearum* in maize (Sekiyama *et al*., 2005).

Deoxynivalenol (DON, vomitoxin) is a mycotoxin that occurs predominantly in grains such as wheat, barley, oats, rye, and maize, and less often in rice, sorghum, and triticale. The occurrence of deoxynivalenol is associated primarily with *F. graminearum* and *F. culmorum*, both of which are important plant pathogens. Deoxynivalenol has been implicated in incidents of mycotoxicoses in both humans and farm animals (Canady *et al*., 2001).

2.4 Control of moulds

One of the available methods of controlling moulds is the use of synthetic chemical preservatives which have been the cause of appearance of resistant micro-organisms, leading to occurrences of emerging food borne diseases (Souza *et al*., 2005). Furthermore, other methods like solar driers are expensive for the small scale farmers. In order to avoid the loss of lives due to mycotoxicoses outbreaks, there is need to improve food and health security through post-harvest food protection. This can be achieved by screening and evaluating alternative antimicrobial agents from plant based sources such as essential oils. A great array of plant species has varied medicinal and antimicrobial potentials. It is well established that some plants contain compounds able to inhibit microbial growth (Naqui *et al*., 1994). These plant compounds can have different structures and different actions when compared with conventional antimicrobials used to control microbial growth (Nascimento *et al*., 2000).

Studies have shown that plants that have essential oils possess antibacterial, antifungal, antiviral, insecticidal and antioxidant properties (Burt, 2004). Essential oil is a concentrated, hydrophobic liquid containing volatile aroma compounds from plants. They are obtained from nearly all parts of the plant. Oils play several essential functions for the plant survival including defense against invaders. Several chemical compounds of relatively complex structures with antimicrobial activity include alkaloids, flavonoids, isoflavonoids, tannins, cumarins, glycosides, terpenes, phenylpropanes and organic acids (Nychas, 1996). Essential oils can be obtained by expression, fermentation or extraction but the method of steam distillation is most commonly used for commercial production (Alan and Cornie, 2005). They are composed chiefly of terpenoids with frequently occurring aromatic compounds arising from the phenylpropanoid pathway (e.g. eugenol and safrole). In some species, alkanes, aliphatic alcohols and ketones may be obtained. They are also considered as a complex mixture of various aroma chemicals. Each of the constituents contributes to the beneficial or adverse effects of the oil (Buchbauer, 1993). These volatile oils are more or less modified during the preparation process. These natural products are used as raw materials in many fields, including perfumes, cosmetics, aromatherapy, phytotherapy, pharmaceuticals, spices and nutrition (Buchbauer, 1993). There are no suitable synthetic substitutes of essential oils available due to the difficult of profiling and mimicking the complex compound mixtures in the volatile oils (Jaime and Teixera, 2004).

Extraction of essential oils is relatively easily adaptable procedure and, therefore, it is advisable to use pure oil as compared to the crude extracts which have low efficacy due to low concentration of the active ingredients and interference from other compounds. Various extraction methods are used in the manufacturing of essential oils, and the method used is normally dependent on what type of botanical material is being used (Jerry, 2009).

2.4.1 Effects of essential oils on microorganisms

Antimicrobial activity of volatile compounds results from the combined effect of direct vapour absorption by microorganisms and indirect effect through the medium that absorbed the vapour (Moleyar and Narasimham, 1986). The vapour absorption on microorganisms is determined by their membrane permeability. In general, the inhibitory action of natural products on mould cells involves cytoplasm granulation, cytoplasmic membrane rupturing and inactivation or synthesis, inhibition of intercellular and extracellular enzymes. These actions can occur in isolate or concomitant way and culminate in inhibition of mycelium germination (Cowan, 1999). Research into the antimicrobial action of monoterpenes suggests that they diffuse into and damage cell membrane structures (Andrews *et al.*, 1980; Uribe *et al.*, 1985). Monoterpenes are lipophilic and by definition will preferentially partition from an aqueous phase into membrane structures. This causes expansion of the membrane, increased fluidity or disordering of the membrane structure and inhibition of membrane embedded enzymes (Sikkema *et al.*, 1995). Some authors have attributed this action to the interaction of their functional groups (especially phenols) with the microbial cell envelope (Lahlou and Berrada, 2001). Bammi *et al.* (1997) demonstrated the action of essential oil of thyme on cell life cycle. The appearance of profound lesions in different microorganisms (*Escherichia coli, Bacillus subtilis,* and *Saccharomyces cerevisiae*) clearly demonstrates the action of this oil (Bouchikhi, 1994).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Sample collection

A total of 30 maize samples categorized as good and mould damaged grain collected randomly from various rural households and markets in Trans-nzoia, Kakamega and Kuria district were used in this study. Sampling was done at random with the assistance of the Local Agricultural officers to identify farmers who were growing maize and willing to participate in the study. In Trans-nzoia, samples were collected from Kitale central and Kiminini divisions. In Kakamega, samples were collected from Kakamega municipality division whereas in Kuria district samples were collected from Kehancha and Masaba divisions (Figure 1). Most of these areas are in mid altitude agro ecological zones with warm and humid conditions which favours development of moulds and production of mycotoxins (Kaaya *et al.*, 2006). These areas have unpredictable rainfall patterns making it difficult for small scale farmers to efficiently dry their produce. Ten samples, each weighing half a kilogram were collected from each district in properly labelled khaki paper bags to minimize saprophytic fungal contamination and transported in a cool box to the laboratory for analysis. Each sample was divided into two portions; one was stored at 4°C and the other at -20°C to avoid further accumulation of mycotoxins.

3.2 Isolation and identification of moulds associated with maize

3.2.1 Isolation of Moulds

Moulds were isolated from the samples using the direct plating technique. For each sample, 20 seeds were picked randomly and surface sterilized by soaking for 1 minute in 2.5% of sodium hypochlorite, and rinsed in three changes of sterile distilled water. The samples were blotted with sterile filter paper and plated (five seeds per plate) on the surface of malt extract agar (MEA) and potato dextrose agar (PDA) containing 7.5% sodium chloride and 33 mg/l of streptomycin sulphate powder. Addition of streptomycin sulphate is very effective in the inhibition of bacteria and fast-growing "spreader" moulds such as *Trichoderma*. Yet, it does not inhibit the growth of other mould species, including the mycotoxin producers. Thus, identification is not compromised (Diba *et al.*, 2007). The plates were incubated at 25°C and monitored daily for fungal growth for up to 14 days. Treatments were replicated four times and the experiment was laid down in a complete randomized design.

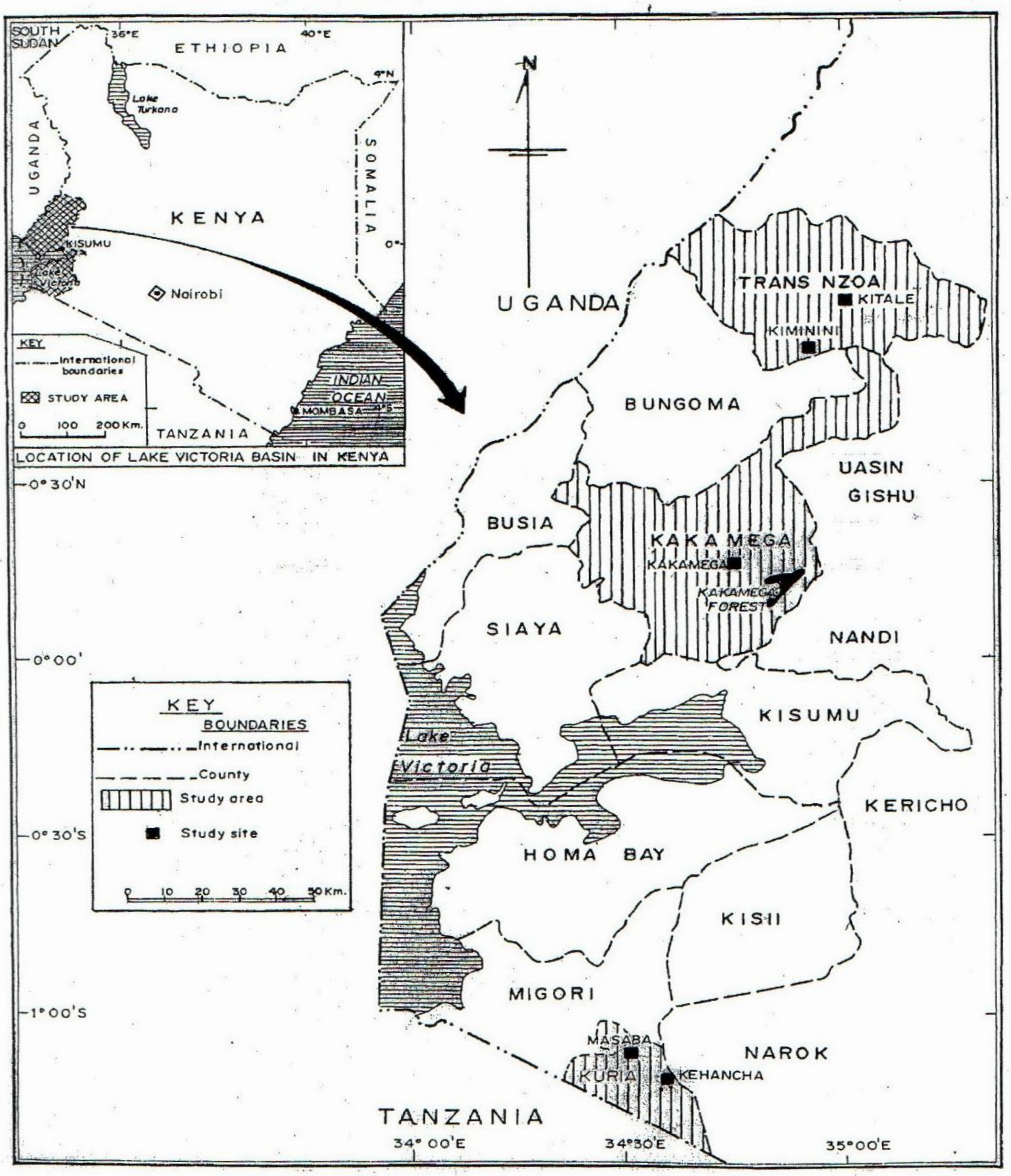

Figure 1 : Map of the study area

Key: Trans-nzoia (Mean Temp: 23°C, RH:37-81%), Kakamega (Mean Temp:26°C, RH: 68-83%) and Migori County (Kuria): Mean Temp:16.5°C, RH :above 80%

Source: Cartographer, Department of Geography, Egerton University (2012)

3.2.2 Identification and quantification of the moulds

The resulting cultures of *Aspergillus* species were identified to species level based on cultural and morphological characteristics using taxonomic keys (Kozakiewtez, 1989; Klich, 2002) and *Fusarium* species were identified based on the criteria of Gerlach and Nirenberg (1982) and Leslie and Summerell (2006). Morphological features of moulds were studied and the major and remarkable macroscopic features that were looked at are colony diameter, colony colour on agar and reverse and colony texture. Microscopic characteristics that helped in the identification process were conidia heads, stipes, colour and length, vesicles shape and seriation, metula covering, conidia size, shape and roughness (Diba *et al*., 2007). *Aspergillus parasiticus* has green colonies but deeper in shade than *A. flavus*. *Aspergillus ochraceus* has ochre-coloured or buff colonies with submerged mycelium; conidiophores show shades of yellow in the outer layer of the wall which is rough or pitted. For *Aspergillus niger,* the hyphae are septate and hyaline more or less yellow in colour. The colonies are black coloured and reverse usually colourless. *Aspergillus versicolor's* cultures show considerable range of colour. Different strains may be variously pale green, grayish green, buff or, show patches of yellow. *Aspergillus nidulans* has colonies of a clear green colour, developing dirty white spots from the centre outwards. Conidiophores are smooth walled, or more or less browned. The number of seeds contaminated by *Aspergillus* and *Fusarium* were counted and the infection rate (number of contaminated grain recorded as percentage) was determined and compared for each sample. Target moulds were sub-cultured to obtain pure cultures and single spore cultures prepared.

Quantification of the moulds was done by determining the microbial loads in the grain. The samples were ground into fine flour in a mill in order to release the fungal inoculum in solution during serial dilutions. Serial dilutions of the flour were made in 0.1% peptone-water and 0.1ml was aseptically surface spread on PDA plates in triplicates (Choi *et al*., 1999). The plates were incubated at 25°C for seven days and the resulting cultures identified based on cultural and morphological characteristics using taxonomic keys (Nelson *et al*., 1983; Kozakiewtez, 1991). The number of colonies of each mould was determined and compared for all the samples.

3.3 Analysis of maize samples for mycotoxins

Maize samples were analyzed for the most common mycotoxins namely; Aflatoxin B_1 (AFB_1) and Aflatoxin G_1 (AFG_1). Thin Layer Chromatography (TLC) was used for the analysis due to its simplicity, speed, ability to handle many samples simultaneously and low costs. Fifty grams of finely ground $\approx$ 20 mesh samples at room temperature was weighed into a flask and extracted with 100 ml distilled water and 300 ml chloroform for 5 min in an omnimixer at a medium velocity as described in Luciana and Eugenia (2001). The extract was then filtered under vacuum through Whatman No. 4 filter paper and the filtrate added 20 g anhydrous sodium sulphate. The filtrate was evaporated in a rotary evaporator (40-50°C), a little chloroform added and then it was transferred to sample bottle. The mixture was spotted near one end of a TLC plate that was coated with a thin layer of silica gel. The standards for AFB_1 and AFG_1 were also spotted. Separation occurred when the end of the plate nearest the spotted mixture was placed in a solvent system at the bottom of a closed vessel and the solvent allowed to migrate through the adsorbent matrix and moved towards the top of the plate. The plates were developed with chloroform: acetone (9:1 v/v) solvent system. The TLC plate was visually examined under U.V light at 365 nm. The analyzed AFG_1 and AFB_1 in the grains were seen as purplish spots under long wavelength ultraviolet light by visual comparison with the standards spotted on the TLC plate.

3.4 Extraction of essential oils from selected aromatic plants

The target aromatic plants that were used in the extraction of essential oils were selected from *Lamiaceae*, *Asteraceae*, *Piperaceae* and *Apiaceae* families which are known to contain antimicrobial essential oils (Matasyoh *et al.*, 2005; 2006; 2007). The essential oils are believed to be safe for human consumption because aromatic plants have traditionally been used by the local people for food preservation. Fresh leaves of *Artemisia vulgare* (Mugwort), *Foeniculum vulgare* (Camphor bush), *Piper capense* (Wild pepper), *Tarchonanthus camphoratus* (Sweet fennel) and *Rosmarinus officinalis* (Rosemary) were collected from Kakamega forest and the nearby fields. The fresh leaves were packed into two litre flasks up to three-quarter full and weighed at 400 gms. About 500 ml of tap water was added and the leaves were subjected to hydro-distillation, using modified Clevenger- type apparatus for 4 hours as described by Matasyoh *et al.* (2008). The resultant mixture of steam and essential oil were passed through a Liebig condenser and cooled by a continuous flow of cold water (Figure 2). Essential oils are less dense than water and were separated as an upper layer, floating on the distillation water. The oil was then collected by decanting into sample bottles and dried using anhydrous sodium sulphate (Na_2SO_4). The procedure was repeated until a sufficient amount of oil for anti-microbial tests was obtained. The oils were stored in a sealed glass vial (Bijoux bottle) at 4°C for further analyses.

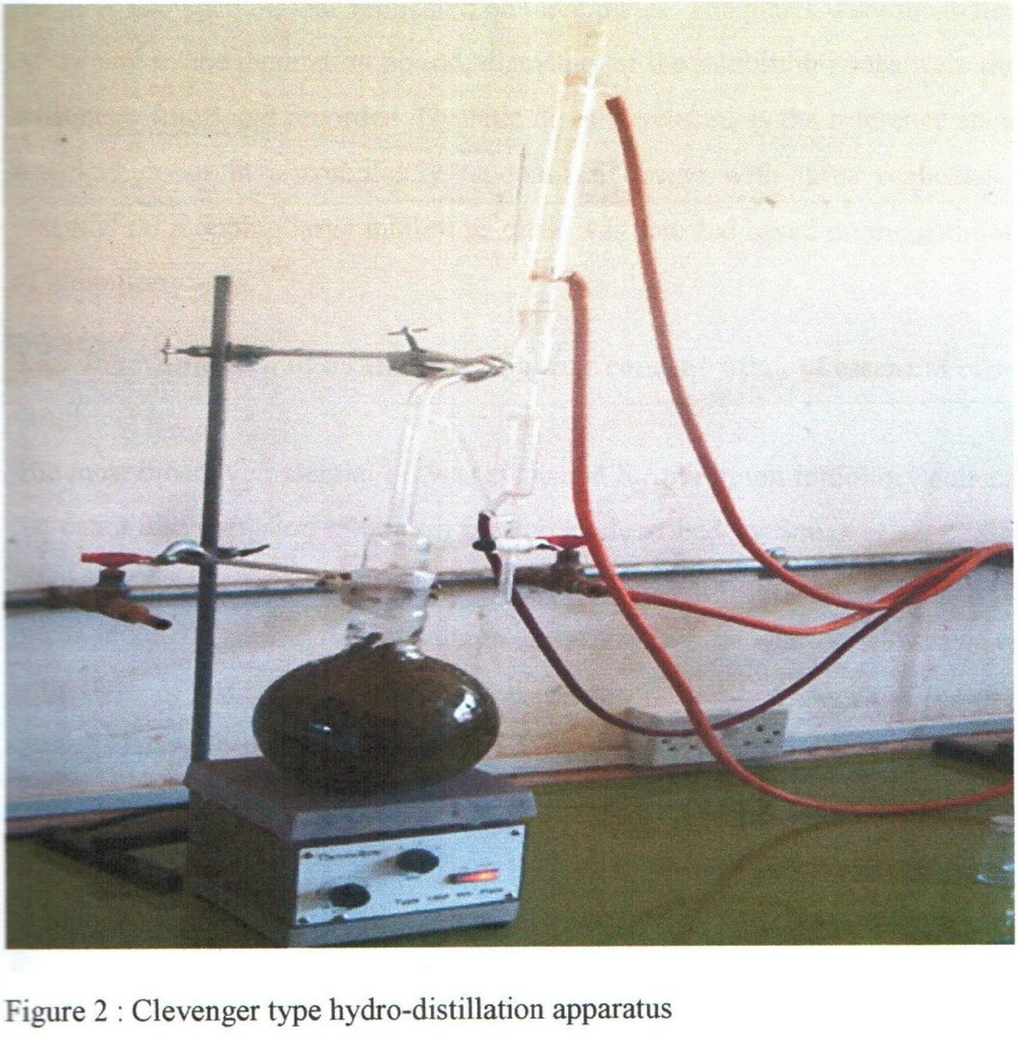

Figure 2 : Clevenger type hydro-distillation apparatus

3.4.1 Screening essential oils for antimould activity

Paper disc diffusion inhibition method was used to screen for antimould activity of the essential oils as described by Souza *et al*. (2005). Five different types of oils (*T. camphoratus, R. officinalis, P. capense, A. vulgare* and *F. vulgare*) were tested for their antimould activity on *A. parasiticus, A.*

flavus, *A. wentii*, *A. ustus*, *A. nidulans*, *A. ochraceus*, *A. tamarii*, *A. niger*, *A. versicolor*, *A. flavipes*, *A. terreus*, *A. fumigatus*, *A. humicola*, *A. sparsus*, *F. solani*, *F. proliferatum*, *F. sporotrichioides*, *F. moniliforme*, *F. scirpi*, *F. chlamydosporum*, *F. trincinctum*, *F. oxysporum*, *F. subglutinans*, *F. nivale*, *F. avenaceum*, *F. graminearum*, *F. culmorum* and *F. crookwellence*. One hundred micro litres of mould suspension (10^6 spores/ml measured using a haemocytometer) prepared with sterile 0.85% physiological saline solution was uniformly spread on sterile solid potato dextrose agar in a Petri dish. Sterile filter paper discs (Whatman No. 1, 6mm in diameter) were soaked with 10 µl of the essential oil and placed at the centre of the inoculated culture plates. One disc was inoculated per plate and each oil was replicated on three plates. The plates were incubated at 25°C for 14 days. At the end of the incubation period, diameters of the inhibition zones were measured to the nearest millimeter (mm) and recorded. Nystatin discs were used as the reference standard. The experiment was laid down in a completely randomized design with three replicates. The most bioactive essential oil inducing large inhibition zones was selected based on the antimould spectrum and size of inhibition zones.

3.4.2 Determination of minimum inhibitory concentration of essential oils on mycotoxigenic fungi

The most bioactive essential oil was evaluated for minimum inhibitory concentrations (MIC) using the paper disc diffusion inhibition method as described by Souza *et al.* (2005). For this, 100 µl of mould suspension prepared as in Section 3.5.1 was uniformly spread on sterile potato dextrose agar media in Petri dishes. After inoculums' adsorption by the agar, sterile sensitivity discs were soaked with 10 µl of the essential oil at various concentrations and placed at the centre of the inoculated culture plates, one disc per plate. Serial dilutions of essential oils were done using 10% dimethyl sulfoxide (DMSO) which was also used as the negative control. The concentration of the original oil extract was 100%. The essential oils were diluted to the following serial geometric dilutions: 50, 25, 12.5, 6.25, 3.13, 1.56 and 0.78. The resultant inhibition zone was used to determine the range for MIC analysis. In all cases, the culture plates were incubated at 25°C for 14 days. The lowest concentration able to induce inhibition zones was considered as the MIC. The experiment was laid down in completely randomized design with three replicates (plates).

3.5 Data analysis

One way analysis of variance (ANOVA) was used to test whether quantity of the moulds were different in the three districts. Least significant difference (LSD) was used to discriminate which maize and from which district was highly contaminated with moulds. Student's t-test was used to test if there was a difference in occurrence of the two genera of moulds from the three districts. The same test was used to test the differences between the maize samples from the three districts in order to ascertain which one had a high concentration of mycotoxins. The statistical level of significance was fixed at $p < 0.05$ (95%). Separation of means for antimould activity was done using LSD and the most bioactive essential oil was selected based on the antimould spectrum and size of inhibition zones. For MIC determination, data was analyzed using SAS software (Proc GLM) and the lowest concentration able to induce inhibition zone was considered as the MIC.

CHAPTER FOUR
RESULTS AND DISCUSSION

4.1 Moulds isolated from the maize and their incidence

Five fungal genera namely *Aspergillus, Fusarium, Penicillium, Rhizopus* and *Mucor* were isolated from the maize samples collected from Trans-nzoia, Kakamega and Kuria districts. *Aspergillus* was the most frequently isolated genus with a 63.3% frequency.

4.1.1 *Aspergillus* species identified

The moulds isolated and identified among the genus *Aspergillus* were 12 mycotoxigenic species which included *A. parasiticus, A. flavus*, *A. wentii*, *A. ustus*, *A. nidulans*, *A. ochraceus*, *A. tamarii, A. niger, A. versicolor, A. flavipes, A. terreus, A. fumigatus,* and two atoxigenic species, *A. humicola* and *A. sparsus* (Table 1). The cultures were identified based on cultural and morphological characteristics using taxonomic keys (Kozakiewtez, 1989; Klich, 2002). Species of the genus *Aspergillus* are found almost everywhere on every conceivable type of substratum (Venkatesh, 2004).

The high occurrence of moulds in the samples can be attributed to the fact that the three districts are found in warm and humid region which predisposes the maize to the moulds in the field and also after being harvested. These findings were also expected because of the relatively high temperature and relative humidity in Lake Basin region, which was optimum for growth of *Aspergillus* species. In all the three districts, the most frequently isolated *Aspergillus* species on maize was *A. flavus,* followed by *A. niger, A. parasiticus* and *A. versicolor* (Table 2). Overall, infection of genus *Aspergillus* on the maize seeds in Trans-nzoia district was substantially higher than those collected from Kuria and Kakamega district. The less prevalent *Aspergillus* species throughout the three districts were *A. fumigatus, A. sparsus, A. tamarii, A. ustus, A. humicola*, and *A. terreus.*

Table 1 : *Aspergillus* and *Fusarium* species isolated from maize samples from Trans-nzoia, Kakamega and Kuria districts

Maize sample	**District/origin**	**Sample Condition**	***Aspergillus* species isolated**	***Fusarium* species isolated**
KEKIM 01	Trans-nzoia	Good	*A. flavus, A. parasiticus, A. wentii, A. terreus*	*F. proliferatum*
KEKIM 02	Trans-nzoia	Bad	*A. flavus, A. parasiticus*	-
KEKIM 03	Trans-nzoia	Good	*A. flavus, A. ochraceus, A. parasiticus, A. niger*	*F. solani*
KEKIM 04	Trans-nzoia	Good	*A. flavus*	*F. solani*
KEKIM 05	Trans-nzoia	Bad	*A. flavus, A. ochraceus, A. wentii*	-
KEKIM 06	Trans-nzoia	Bad	*A. flavus, A. versicolor*	*F. solani, F. moniliforme*
KEKIM 07	Trans-nzoia	Bad	*A. versioclor*	*F. solani*
KEKIM 08	Trans-nzoia	Good	*A. ustus*	-
KEKIM 09	Trans-nzoia	Good	*A. parasiticus*	-
KEKIM 10	Trans-nzoia	Bad	*A. parasiticus*	*F. solani*
KEKAM 18	Kakamega	Good	*A. versicolor*	*F. scirpi*
KEKAM 19	Kakamega	Good	*A. flavipes*	-
KEKAM 20	Kakamega	Good	*A. flavus, A. niger*	*F. chlamydosporum*
KEKAM 21	Kakamega	Good	*A.flavus, A. niger, A. fumigatus*	*F. graminearum*
KEKAM 22	Kakamega	Good	*A. wentii*	-
KEKAM 23	Kakamega	Good	*A. ustus*	*F. trincinctum, F.*

				chlamydosporum, F. semitectun, F. oxysporum
KEKAM 24	Kakamega	Bad	*A. nidulans, A. terreus*	*F. nivale, F. proliferatum, F.chlamydosporum*
KEKAM 25	Kakamega	Good	-	*F. graminearum*
KEKAM 26	Kakamega	Good	-	*F. avenaceum, F. subglutinans*
KEKAM 27	Kakamega	Bad	*A. flavus, A. parasiticus*	-
KEKUM 31	Kuria	Bad	*A. flavus, A. versicolor*	*F. merismoides, F. culmorum*
KEKUM 32	Kuria	Good	*A. flavus, A. ochraceus*	*F.crookwellence*
KEKUM 33	Kuria	Good	*A. ustus, A. wentii, A. parasiticus, A. sparsus*	*F. proliferatum, F. nivale*
KEKUM 34	Kuria	Good	*A. flavus, A. niger*	*F. proliferatum*
KEKUM 35	Kuria	Good	*A. humicola*	*F. sporotrichioides*
KEKUM 36	Kuria	Bad	*A. niger, A. nidulans*	*F. merismoides*
KEKUM 37	Kuria	Bad	*A. niger*	*F. merismoides*
KEKUM 38	Kuria	Good	*A. flavus, A. tamari, A. sparsus, A. ochraceus*	*F. chlamydosporum, F. merismoides*
KEKUM39	Kuria	Bad	*A. flavus, A. nidulans*	*F. oxysporum*
KEKUM 40	Kuria	Good	*A. flavus, A. nidulans, A. versicolor, A. fumigatus*	*F. proliferatum, F. solani*

Legend for Maize Sample Codes

KEKIM: KE-Kenya	KI- Kitale (Trans-nzoia)	M-Maize
KEKAM: KE-Kenya	KA- Kakamega	M-Maize
KEKUM: KE-Kenya	KU- Kuria	M-Maize

Table 2: Percentage occurrence of *Aspergillus* species in Trans-nzoia, Kakamega and Kuria districts

***Aspergillus* species**	**Trans-nzoia**	**Kakamega**	**Kuria**
A. flavus	23.1	15	20
A. parasiticus	15.4	10	8
A. wentii	7.7	0	8
A. niger	3.8	15	15
A. ochraceus	7.7	5	4
A. versicolor	7.7	15	7
A. flavipes	3.8	10	5
A. terreus	3.8	0	4
A. nidulans	3.8	10	4
A. ustus	7.5	5	12
A. sparsus	7.5	0	8
A. fumigatus	3.6	10	0
A. humicola	3.6	5	0
A. tamarii	0	0	1

The percentage occurrence of *Aspergillus* population was determined by comparing the number of seeds showing each type of mould growth for each sample. When the *Aspergillus* population was separated according to geographical areas of Western Kenya, the predominant *Aspergillus* species in Trans-nzoia was *A. flavus* (23.1%) followed by *A. parasiticus* (15.4%), *A. wentii* (7.5%), *A. ochraceus* (7.5%), *A. versicolor* (7.5%), *A. ustus* and *A. sparsus* at 7.5%. *Aspergillus flavipes, A. nidulans, A. niger* and *A. terreus* were the least isolated species at a frequency of 3.8%, while *A. fumigatus* and *A. nidulans* were at 3.6%. In Kakamega district, the most prevalent *Aspergillus* species were *A. flavus, A. niger* and *A. versicolor* at 15%, followed by *A. parasiticus, A. flavipes, A. nidulans* and *A. fumigatus* at 10%, *A. ochraceus, A. humicola* and *A. sparsus* at 5%. In Kuria district, the most frequent *Aspergillus* species isolated were *A. flavus* (20%), followed by *A. niger* (15%) and *A. parasiticus, A. wentii, A. sparsus,* and *A. humicola* were isolated at 8 %.

The ubiquitous nature of the moulds, ability to colonize diverse substrates and lack of effective control measures (Souza *et al.*, 2005) could have contributed to the high incidences of the moulds in maize isolated from these regions. *Aspergillus* species are more commonly associated with cereals during drying and storage. *Aspergillus flavus* and *A. parasiticus* have a particular affinity for cereals and can be recognized by yellow-green or grey green colour on corn kernels in the field and in storage (Varga *et al.*, 2011). Pre-harvest invasion is partly dependent on insect damage to cobs, but the fungi can also invade down the silks of developing ears and they cause diseases like maize ear and kernel rot. Invasion is primarily due to inadequate drying and improper storage (Pitt, 2000). There is a possibility that since invasion starts in the field and continues in storage, the maize samples collected from markets and various rural households had already accumulated high levels of the moulds especially the *A. flavus* followed by *A. parasiticus* because they commonly attack maize both in the field and in storage.

Damaged maize also favors the growth of *A. flavus* compared to any other *Aspergillus* species and this fact explains why the most frequently isolated *Aspergillus* species in the three districts, was *A. flavus* followed by *A. parasiticus* and other *Aspergillus* species. According to Fandohan *et al.* (2003), postharvest handling favourably and unfavourably affects fungal infection and mycotoxin production in maize. Mechanical damage during and after harvest may also offer entry to the fungal spores either in maize cobs or grains. This possibly explains why very high quantities of the moulds were isolated from the maize samples because some of them had damaged grains that maybe predisposed the grains to the fungus infection.

Fields that vary in cropping history, tillage practices, planting dates, soil types or hybrids can differ greatly in mould and aflatoxin contamination (Munkvold *et al.*, 2009). There is likelihood that cropping history, tillage practices, soil types, planting dates and hybrids planted in the three districts are different. These factors may explain the differences in the quantity and type of the moulds isolated from the three districts.

4.1.2 *Fusarium* species identified

Maize was found to be the host of an extremely wide range of *Fusarium* species under natural infection at the sample sites examined within the Lake basin region. Among the genus *Fusarium,* sixteen mycotoxigenic species were identified and these included *F. solani*, *F. proliferatum*, *F. sporotrichioides, F. moniliforme, F. scirpi, F. chlamydosporum, F. trincinctum, F. oxysporum*, *F. subglutinans, F. merismoides, F. semitectum, F. nivale, F. avenaceum, F. graminearum, F. culmorum* and *F. crookwellence* (Table 1). In Trans-nzoia district, the most frequent *Fusarium* species was *F. solani* at 71.4%, followed by *F. proliferatum*, *F. avenaceum*, *F. sporotrichoides,* and *F. moninliforme* at 7.15% as shown in Table 3. In Kakamega district, *F. chlamydosporum* was the highest at 25.1% followed by *F. graminearum* at 16.7%, *F. oxysporum, F. nivale, F. proliferatum, F. subglutinans, F. semitectum,* and *F. trincinctum* at 8.3%, *F. crookwellence* was at 4.3% and *F. scirpi* the least isolated at 4%. The most prevalent *Fusarium* in Kuria district was *F. merismoides* at 36.4% followed by *F. proliferatum* at 27.3%, *F. oxysporum, F. solani* and *F. nivale* at 9.1%, *F. chlamydopsorum* at 5.1% and *F. culmorum* at 4.3%.

Table 3: Percentage occurrence of *Fusarium* species in Trans-nzoia, Kakamega and Kuria districts

***Fusarium* species**	**Trans-nzoia**	**Kakamega**	**Kuria**
F. solani	71.4	0	9.1
F. merismoides	0	0	36.4
F. nivale	0	8.3	9.1
F. proliferatum	7.15	8.3	27.3
F. oxysporum	0	8.3	9.1
F. chlamydosporum	0	25.1	5.1
F. graminearum	0	16.7	0
F. subglutinans	0	8.3	0
F. semitectum	0	8.3	0
F. avenaceum	7.15	0	0
F. trincinctum	0	8.3	0
F. scirpi	0	4	0
F. culmorum	0	0	4
F. crookwellence	0	4.3	0
F. sporotrichioedes	7.15	0	0

F. moniliforme	7.15	0	0

All the *Fusarium* species isolated and identified are considered to be pathogenic to maize. This is in line with observations of Munkvold (2003) and the statement of Leslie and Summerell (2006) that *Fusarium* species is the most common pathogen on maize cobs. *Fusarium* species are destructive pathogens on cereal crops and other commodities, and produce mycotoxins before, or immediately after, harvest (Pitt, 2000). They are commonly considered as field fungi invading more than 50% of maize grains before harvest (Robleda-Robleda, 1991).

Generally when *Fusarium* species invade maize in the field they cause diseases like seedling blights, kernel, root, seed, stalk and ear rots. For example *F. subglutinans* and *F. moniliforme* cause Fusarium ear rot and stalk rots. *Fusarium graminearum* attack maize and cause stalk, cob and root rots. *Fusarium moniliforme* also causes many other diseases like kernel and root rot, seed rot and seedling blight (CIMMYT, 2004). Reports of surveys conducted in some African countries showed *F. moniliforme* as the most prevalent fungus on maize ((Marasas *et al.,* 1988; Allah Fadi, 1998; Kedera *et al.*, 1999). However, this was not true with the findings of the current study because the most frequently isolated *Fusarium* were *F. proliferatum, F. solani, F. chlamydosporum, F. merismoides* and *F. nivale.*

Sixteen *Fusarium* species were isolated from the three districts within the Lake Basin region (Table 3). The high number of species isolated could be due to interaction among fungi in maize, which constitutes an important factor influencing fungal infection and subsequent mycotoxins production. Mechanical damage during and after harvest may also offer entry to the fungal spores either in maize cob or grains. Grain damage is a common occurrence in maize especially during postharvest handling of the grains. This possibly predisposes the maize to *Fusarium* attack within the region ((Fandohan *et al.*, 2003). Another factor that could explain the high level of the *Fusarium* within the three districts is insect invasion. Insects also play an important role in infection of maize by *Fusarium* species. They can act as wounding agents or as vectors spreading the fungus from origin of inocula to plants. Insects are a great havoc to maize in the field as well as in the store and many pests and parasites attack maize during the storage period. According to Gwinner *et al.* (1996), insects are most often considered as the principal cause of grain losses. This therefore means that fungi always invade wherever insects have attacked maize and it justifies the high quantities of *Fusarium* species isolated from the three districts. The occurrence, prevalence and diversity of *Fusarium* species did not vary substantially between the samples.

Generally, the relative level of *Fusarium* distribution within and between the districts ranged from 0% to 71.4%. However, the distribution of the moulds isolated and identified from the three districts were not significantly different at P≤0.05 as shown in Table 4. When the data of the moulds isolated in the three districts was analyzed separately using Tukey HSD, there was no significant (P≤0.05) difference in the number of the moulds in the three districts. The mean difference in the number of the moulds between Trans-nzoia and Kakamega was 0.365, between Trans-nzoia and Kuria was 0.335 and between Kuria and Kakamega was 0.030 (Table 5).

Table 4: Analysis of variance for significance of moulds distribution within and between the three districts

Number of moulds					
Source of variation	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	2.014	2	1.007	.846	.434
Within Groups	80.944	68	1.190		
Total	82.958	70			

df=degree of freedom, F= Frequency, Sig=Significance

Table 5: Comparison of the moulds in Trans-nzoia, Kakamega and Kuria districts

Multiple Comparisons

Turkey HSD

(I) Sampling place	(J) Sampling place	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Kakamega	Trans-nzoia	-.365	.325	.502	-1.14	.41
	Kuria	-.030	.327	.995	-.81	.75
Trans-nzoia	Kakamega	.365	.325	.502	-.41	1.14
	Kuria	.335	.306	.519	-.40	1.07
Kuria	Kakamega	.030	.327	.995	-.75	.81
	Trans-nzoia	-.335	.306	.519	-1.07	.40

4.2 Quantification of moulds in the maize samples

The moulds were quantified to determine the microbial loads in the grain. The number of colonies of each mould was determined and compared for all the samples. The moulds microbial load was compared in each district using Turkey HSD assuming that the variances are equal. There was a significant ($P \leq 0.05$) difference in the load between any two districts (Table 6). The mean difference in the quantity of moulds between Trans-nzoia and Kuria was higher (72.4 with a STD error of 9.8) as compared to Trans-nzoia and Kakamega districts at 40.5 with an error of 10.7 at P>5% confidence level. The mean difference in the number of moulds between Kakamega and Kuria was 31.9 at P>5% confidence level. The moulds isolated from mouldy and good maize were compared using T- test for each of the districts. The results showed that the quantity of the moulds between good and mouldy maize were not significantly ($P \leq .0.05$) different. The mean numbers of colonies for good and mouldy maize were compared using T-test. The results showed a very minimal difference in the number of colonies for both good and mouldy maize (Table 7).

Table 6: Comparison of the mean number of mould colonies between the districts

Multiple Comparisons

Mean number of colonies Turkey HSD

(I)	(J)				95% Confidence Interval	
District	District	Mean Difference (I-J)		Sig.	Lower Bound	Upper Bound
Trans-nzoia	Kuria	72.38194*	9.82438	.000	48.9650	95.7988
	Kakamega	40.46528*	10.65603	.001	15.0661	65.8645
Kuria	Trans-nzoia	-72.38194*	9.82438	.000	-95.7988	-48.9650
	Kakamega	-31.91667*	10.91918	.012	-57.9431	-5.8902
Kakamega	Trans-nzoia	-40.46528*	10.65603	.001	-65.8645	-15.0661
	Kuria	31.91667*	10.91918	.012	5.8902	57.9431

*Mean difference between any two districts

Table 7: Comparison between the mean number of colonies between good and mouldy maize

T-Test: Good and bad maize by district

Group Statistics

District		Condition of maize	Std. Deviation	Std. Error Mean
Trans-nzoia	Mean number of colonies	Good	49.16006	10.72761
		Bad	41.91028	10.82119
Kuria	Mean number of colonies	Good	30.62881	7.65720
		Bad	31.37540	7.84385
Kakamega	Mean number of colonies	Good	46.01233	10.28867
		Bad	22.39559	11.19780

Relatively high quantities of the moulds were detected from each of the districts sampled. These findings were expected because of the relatively high temperature and relative humidity in Lake Victoria Basin, which predisposes the maize to high infections of the moulds. There was a significant difference in the quantity of moulds between the three districts. In addition, differences were observed in the types of moulds occurring in the three districts probably due to use of different maize varieties by farmers in the respective districts. This is because maize hybrids vary in their resistance to the various types of ear molds (Munkvold *et al.*, 2009). Some hybrids are more susceptible to ear mold than others and most probably the maize varieties planted in Trans-nzoia are different from those planted in Kakamega and Kuria. This therefore explains the difference in the type and quantity of moulds in the three districts. Environmental conditions also lead to localized or widespread outbreaks of ear molds that can produce mycotoxins contamination. Factors that affect ear mold development can vary from one portion of a field to another and include previous crop, tillage practices and even small temperature, moisture and humidity differences due to differences in elevation, slope and air movement (Munkvold *et al.*, 2009). These three environmental factors determine the direction to which the spores of the ear mold will be blown and their mass. This could have a direct effect on the accumulation of the fungus on the grain. Farmers, therefore, need to be trained on improved pre-harvest and post-harvest practices to decrease the likelihood of mould infection. From the results obtained, there was no difference in the quantity of moulds in the mouldy and good maize indicating that maize which appears good is also contaminated with moulds.

4.3 Aflatoxin analysis of maize samples

The maize extracts were spotted on a TLC plate together with the standards and presence of aflatoxins detected by visual analysis (Figures 3 - 5). The best separation of extracts was obtained by developing the TLC plate with chloroform: acetone (9:1, v/v, saturated tank) giving an easily recognized profile. Out of the 30 maize samples that were tested for aflatoxins, 84.6% were positive for aflatoxins and 15.4% of samples did not have any detectable amount of the aflatoxins. A high percentage (83.8 %) of samples contained the B_1 aflatoxins only, and 9.7% of samples had G_1 aflatoxins alone. Only 6.5 % of isolates had both B_1 and G_1 aflatoxins. From Trans-nzoia district, samples coded KEKIM 1, 2, 3,4, 6, 7, 12 and 13 had B_1 aflatoxins only and not G_1 whereas KEKIM 8, 9, 10 had G_1 aflatoxins only. None of the samples from Trans-nzoia had both B_1 and G_1 aflatoxins (Table 4). In Kakamega district, ten samples were analyzed and out of these, nine samples coded KEKAM 18, 20, 21, 22, 23, 24, 25, 26, 27 and 28 contained B_1 aflatoxins (Table 8). Only one sample coded KEKAM 27 that was mouldy maize had both G_1 and B_1 aflatoxins. From Kuria district, ten samples out of twelve analyzed were positive for aflatoxins. The nine samples coded KEKUM 31, 32, 33, 34, 35, 37, 38, 41 and 43 contained B_1 aflatoxins only. KEKUM 37 had both B_1 and G_1 aflatoxins. KEKUM 39 had G_1 aflatoxins only. Two maize samples from Kuria district coded KEKUM 36 and 42 did not contain detectable amounts of any of the toxins when analyzed by TLC. The two samples had been isolated from mouldy maize.

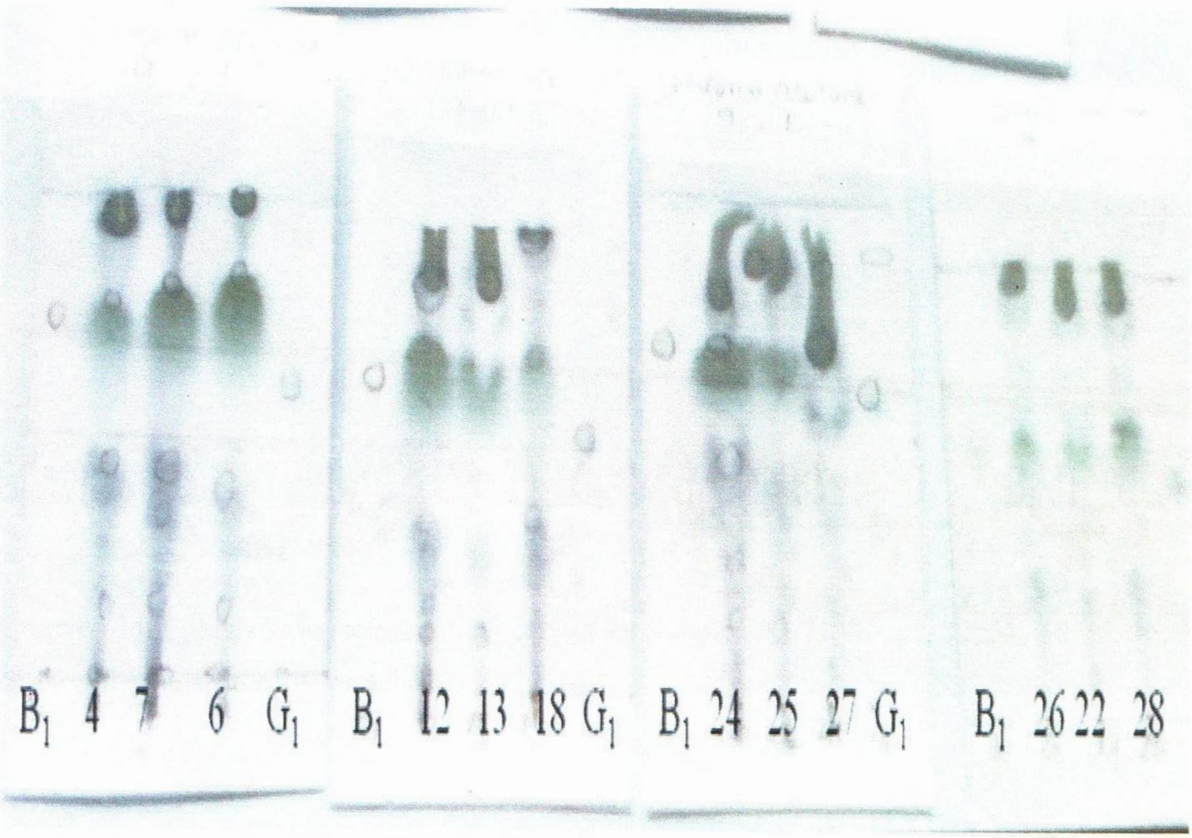

Figure 3: TLC plates showing aflatoxin analysis for samples 4, 6, 7, 12, 13, 18, 22, 24, 25, 26, 27, 28 and standards AFG_1 and AFB_1.

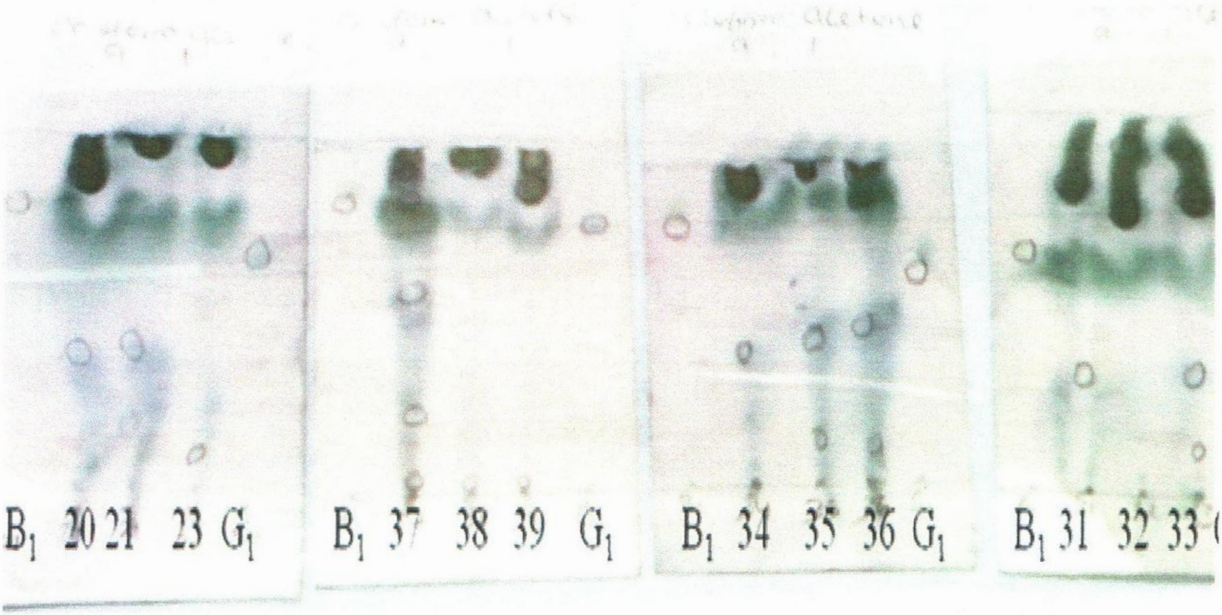

Figure 4 : TLC plates showing aflatoxin analysis for samples 20, 21, 23, 31, 32, 33, 34, 35, 36, 37, 38, 39 and standards AFB_1 and AFG_1.

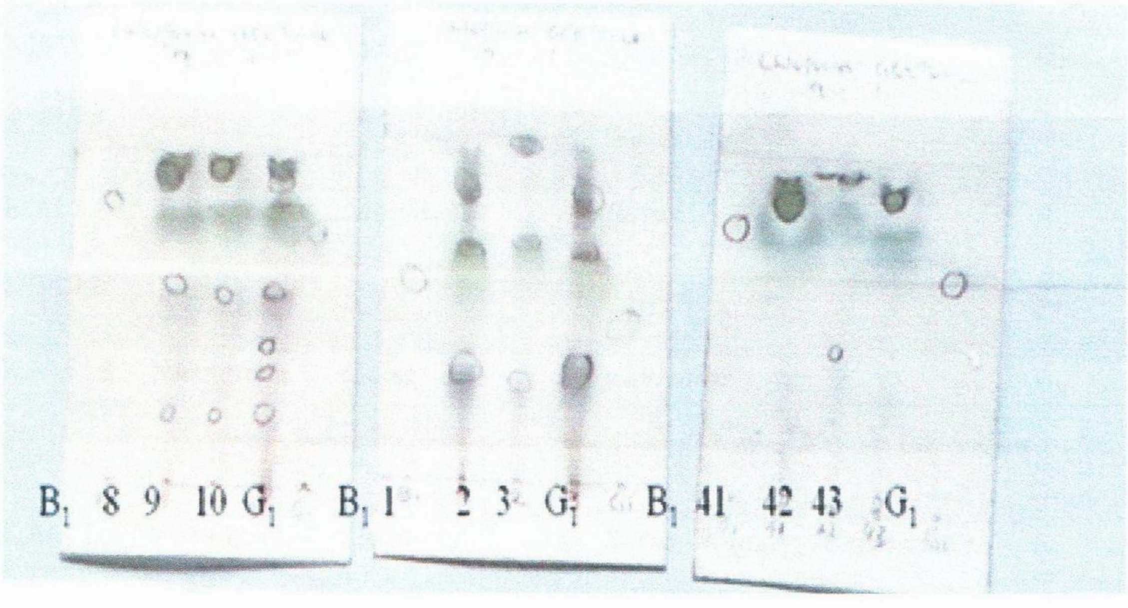

Figure 5: TLC plates showing aflatoxin analysis for samples 1, 2, 3, 8, 9, 10, 41, 42, 43 and standards AFB_1 and AFG_1.

Table 8: Source of maize samples, their conditions, aflatoxigenic moulds isolated and aflatoxins detected by TLC from each sample

Maize sample	District	Condition	Aflatoxigenic moulds isolated	Aflatoxins detected
KEKIM 01	Trans-nzoia	Good	*A. flavus, A. wentii, A. flavipes, A. terreus*	B_1
KEKIM 02	Trans-nzoia	Mouldy	*A. flavus, A. parasiticus*	B_1
KEKIM 03	Trans-nzoia	Good	*A. flavus, A. niger, A. parasiticus, A. ochraceus*	B_1
KEKIM 04	Trans-nzoia	Good	*A. flavus*	B_1
KEKIM 06	Trans-nzoia	Mouldy	*A. flavus*	B_1
KEKIM 07	Trans-nzoia	Mouldy	*A. versicolor*	B_1
KEKIM 08	Trans-nzoia	Good	*A. flavus*	G_1
KEKIM 09	Trans-nzoia	Good	*A. parasiticus*	G_1
KEKIM 10	Trans-nzoia	Mouldy	*A. parasiticus*	G_1
KEKIM 12	Trans-nzoia	Mouldy	*A. flavus*	B_1
KEKIM 13	Trans-nzoia	Good	*A. parasiticus*	B_1
KEKAM 18	Kakamega	Good	*A. versicolor*	B_1
KEKAM 20	Kakamega	Good	*A. flavus, A. niger*	B_1
KEKAM 21	Kakamega	Good	*A. flavus, A. niger, A. fumigatus*	B_1
KEKAM 22	Kakamega	Good	*A. flavus, A. parasiticus*	B_1
KEKAM 23	Kakamega	Good	*A. flavus, A. niger*	B_1

KEKAM 24	Kakamega	Mouldy	*A. flavus, A. parasiticus*	B_1
KEKAM 25	Kakamega	Good	*A. ochraceus*	B_1
KEKAM 26	Kakamega	Good	*A. flavus*	B_1
KEKAM 27	Kakamega	Mouldy	*A. flavus*	B_1 and G_1
KEKAM 28	Kakamega	Good	*A. flavus, A. ochraceus, A. niger*	B_1
KEKUM 31	Kuria	Mouldy	*A. flavus, A. niger*	B_1
KEKUM 32	Kuria	Good	*A. flavus, A. ochraceus*	B_1
KEKUM 33	Kuria	Good	*A. ustus, A. wentii, A. sparsus A. parasiticus,*	B_1
KEKUM 34	Kuria	Good	*A. flavus, A. niger*	B_1
KEKUM 35	Kuria	Good	*A. flavus, A. parasiticus*	B_1
KEKUM 36	Kuria	Mouldy	*A. niger, A. nidulans*	No
KEKUM 37	Kuria	Mouldy	*A. niger, A. flavus*	B_1 and G_1
KEKUM 38	Kuria	Good	*A. flavus, A. tamarii, A. sparsus, A. ochraceus*	B_1
KEKUM 39	Kuria	Mouldy	*A. versicolor, A. flavus, A. nidulans, A. fumigatus*	G_1
KEKUM 41	Kuria	Mouldy	*A. humicola, A. flavipes*	B_1
KEKUM 42	Kuria	Mouldy	*A. versicolor*	No
KEKUM 43	Kuria	Good	*A. sparsus, A. humicola, A. fumigatus, A. versicolor*	B_1

Although aflatoxins are not automatically produced whenever grains become mouldy, the risk of aflatoxin contamination is greater in damaged, mouldy grain than in grains with little mould (Munkvold *et al.*, 2009). However, there is still no firm understanding of why contamination occurs during certain years, but not in others. In this regard, the conflicting involvements of insect damage to the crop, drought, and natural microbiological competition in creating favorable conditions for aflatoxin contamination complicate research efforts (Perrone *et al.*, 2007). There is a possibility of microbiological competition that explains the absence of aflatoxins in the two mouldy samples. There is also a possibility that some of the *A. flavus* strains identified from the samples were atoxigenic strains and thus the absence of the aflatoxins in the samples.

Of the 11 maize samples from Trans-nzoia district analyzed for aflatoxins, 72.7% contained the B_1 aflatoxins and only 27.3% had the G_1 aflatoxins. Likewise, 90% of all the samples from Kakamega district had B_1 aflatoxins and 10% had G_1 aflatoxins. In Kuria district, 58.3% contained the B_1 aflatoxins, 16.7% had the G_1 aflatoxins, 8.3% had both the B_1 and G_1 aflatoxins and 16.7% did not contain any detectable amount of aflatoxins.

In Trans-nzoia district, *A. flavus* was the predominant species at a frequency of 23.1% followed by *A. parasiticus* at 15.4%. In Kuria and Kakamega districts, the same trend was observed with *A. flavus* being the most dominant at 20% and 15% respectively. The most predominant aflatoxins B_1 and G_1 were detected from the samples because the most dominant species isolated from the three districts were *A. flavus* and *A. parasiticus* which are the chief producers of B_1 and G_1 aflatoxins. These findings were therefore expected because of the relatively high temperature and relative humidity in the sampling region, which was optimum for growth of *A. flavus* and *A. parasiticus* and biosynthesis of aflatoxins. Aflatoxins B_1 and G_1 produced primarily by the fungi *A. flavus* and *A. parasiticus* are very potent carcinogens in both humans and livestock and can readily contaminate maize grain in the field and in storage (Munkvold *et al.*, 2009). `

Aflatoxin contamination is higher in maize that has been produced under stress conditions (Atehnkeng *et al*, 2007). Stress on developing maize, particularly during reproductive growth, facilitates infection by the fungi, production of mycotoxins and contamination of the grain. Drought, excessive heat, inadequate plant nutrition, insect feeding on developing kernels, weeds, excessive plant populations, and other plant diseases can produce plant stress and facilitate the infection of maize grain by mycotoxin producing fungi (Laura and Allen, 2009). However, a number of factors, including variety, time, growth conditions and storage conditions of the maize may have contributed to the absence of detectable amounts of aflatoxins in some of the samples. Hybrids genetically engineered to resist insects have been shown to have lower levels of aflatoxins (USDA, 2004). Timely planting of adapted hybrids, proper plant nutrition, irrigation, and insect control either by insecticides or the use of transgenic hybrids all assist in curbing mycotoxin contamination (Hell, 2003). Farmers therefore need to be trained on improved pre-harvest and post-harvest practices in decreasing the likelihood of mould infection and mycootxin contamination. Aflatoxin B_1 was the most predominant type and was found to contaminate maize kernels from all the three districts. These results indicate that consumers of maize produced in the three districts of Kenya are exposed to the dangers of aflatoxin poisoning. Thus, there is the need for policy makers to establish and enforce maize quality standards and regulations related to moulds and aflatoxins across the region to minimize health hazards related to consumption of contaminated kernels.

4.4 Screening Essential oils for antimould activity

All the five essential oils extracted from *Tarchonanthus camphoratus*, *Rosmarinus officinalis*, *Artemisia vulgare*, *Foeniculum vulgare* and *Piper capense* were screened for their activity and they presented inhibitory action on 14 *Aspergillus* and 13 *Fusarium* species assayed. Screening results for antimould activity of the oils on the mould strains are shown in Table 9 and 10. The essential oil of *R. officinalis* had good activity against the *Aspergillus* species. The best result was exhibited on *A. nidulans* with an inhibition zone of 18.3 mm. The inhibition is considered high compared to the standard Nystatin that had an inhibition of 20 mm in diameter. The oil showed least activity on *A. niger* with a diameter of 6.3 mm. The oil did not show any inhibition on *A. tamarii*.

For *Artemisia vulgare*, the best results were on *A. nidulans* with a zone of 21.3 mm which was higher compared to Nystatin (20 mm). This was followed by activity on *A. fumigatus* at 11.7 mm. Least inhibition was exhibited on *A. parasiticus, A. flavus, A. humicola* and *A. ustus*. The oil did not show any antifungal activity against *A. ochraceus, A. tamarii, A. flavipes, A. wentii, A. terreus* and *A. versicolor*.

The essential oil of *Foeniculum vulgare* had good antifungal activity against *A. nidulans* at 12.3 mm. Though this is low compared to the standard (Nystatin = 20 mm), it was the highest zone of inhibition by this oil. Least antifungal activity was shown on *A. wentii* at 6 mm and no activity was exhibited on *A. parasiticus*. *Piper capense* had good activity on *A. fumigatus* with an inhibition zone of 18.3 mm, followed by *A. niger* at 13.7 mm. The least activity was exhibited on *A. flavus* at a diameter of 6.7 mm. There was no activity against *A. parasiticus, A. tamari, A. nidulans* and *A. versicolor*. For *Fusarium* species, *R. officinalis* had good activity against *F. trincinctum* at 17.3 mm and the least activity was exhibited on *F. graminearum* at 6.3 mm. Though in some species there was minimal activity, the oil had activity on all the *Fusarium* species tested. *Artemisia vulgare* had good activity on *F. semitectum* at 15.7 mm and least activity was exhibited on *F. graminearum* and *F. moniliforme.* The oil had activity on all the *Fusarium* species screened. The essential oil of *Foeniculum vulgare* had good activity on *F. semitectum* and least activity on *F. solani* and *F. trincinctum.* No antifungal activity was exhibited by the oil on *F. avenaceum* and *F. moniliforme. Piper capense* had good activity on *F. nivale* at 14.3 mm. This zone was higher than the standard which was 10 mm. The least activity was on *F. avenaceum* and *F. scirpi* at 6.7 mm. However, the oil had activity against all the *Fusarium* species screened.

Tarchonanthus camphoratus essential oil was the most active against *Aspergillus* and *Fusarium* species. The best results for *T. camphoratus* were on *A. nidulans* at an inhibition zone of 14.3 mm in diameter followed by *A. fumigatus* (12.3 mm) and the least antifungal activity of the oil was observed against *A. terreus* with an inhibition zone of 6.7 mm in diameter. The highest activity of the oil on the genus *Fusarium* was observed against *F. oxysporum* with the largest inhibition zone of 24.3 mm as compared to 11 mm inhibition by Nystatin. This was followed by activity against *F. solani* (21.3 mm) and lastly *F. nivale* at (11.3 mm). The oil was therefore selected after screening all the five for further analysis of its Minimum Inhibitory concentration.

Literature shows that *R. officinalis* has antimicrobial activities against a variety of bacteria, fungi, moulds and viruses (Salehi *et al.*, 2007). The antimicrobial activity of the oil was tested on various microorganisms and it showed promising antimicrobial activity according to Antony (2007). This explains why the oil had good antimould activity against the *Aspergillus* and *Fusarium* species screened in this study. *Artemisia vulgare* oil has been evaluated and results presented a great variety of sesquiterpenes that could be considered answerable for its antimicrobial activity. Although they usually occur as complex mixture, their activity may generally account for in terms of their major components (Glauciemar *et al.*, 2009). The oil of *F. vulgare* has been shown to be effective against fungal pathogens causing diseases in plants and human beings according to Sunita and Mahendra (2008). According to their findings, the oils are important source of fungi toxic compounds and they may provide a renewable source of useful fungicides that can be utilized in antimycotic drugs against *A. fumigatus* and *A. niger*. The results support the notion that plant essential oils have a role as pharmaceuticals and preservatives. The good activity of *Piper capense* owes to their leaves having a high content of sesquiterpenes (65.2-89.5%) (Tchoumbougnang *et al.*, 2009). The most prominent compound in the leaves was found to be α- pinene (12.8%) and β-pinene (50.1%). Consequently research shows that the oil is quantitatively rich in monoterpenes (80.8%). The activity of this oil against *Aspergillus* and *Fusarium* species can be attributed to the monoterpenes found in the oil. The result is similar to one done in Cameroon where *P. capense* was shown to possess high levels of insecticidal activity.

Table 9: Inhibition zones (mm) induced by essential oils of *Tarchonanthus camphoratus, Rosmarinus officinalis, Artemisia vulgare, Foeniculum vulgare* and *Piper capense* on *Aspergillus* species isolated from maize samples after 14 days of incubation

Mould species	**Essential oils[a]**						
	T. camphoratus	***R. officinalis***	***A. vulgare***	***F. vulgare***	***P. capense***	**STD[b]**	**Control**
A. flavus	8.0	7.7	6.3	7.0	6.7	12	0
A. ochraceus	10.7	7.0	0	9.0	8.7	17	0
A. parasiticus	8.3	9.7	6.3	0	0	14.5	0
A. niger	10.7	6.3	6.0	10.3	13.7	20.5	0
A. tamarii	10.2	0	0	8	0	13	0
A. fumigatus	12.3	13.0	11.7	10.7	18.3	20.5	0
A. flavipes	9.8	6.3	0	6	10	20.5	0
A. ustus	10.3	6	6.3	8.3	7.3	15.5	0
A. nidulans	14.3	18.3	21.3	12.3	0	20	0
A. wentii	8.5	5.7	0	5.7	10.7	16	0
A. sparsus	10.2	7.3	8.7	12	9.3	13.3	0
A. versicolor	9.3	8.0	0	8.7	0	19.3	0
A. terreus	6.7	6.3	0	7.3	6	18.3	0
A. humicola	8.3	7	6.3	10.3	7	12.3	0

[a] Absolute concentration; [b] Nystatin Control: Pure culture with no disc

Table 10: Inhibition zones (mm) induced by essential oils of *Tarchonanthus camphoratus, Rosmarinus officinalis, Artemisia vulgare, Foeniculium vulgare and Piper capense* on *Fusarium* species isolated from maize samples after 14 days of incubation

Mould species	**Essential oils**[a]						
	T. camphoratus	***R. officinalis***	***A. vulgare***	***F. vulgare***	***P. capense***	**STD**[b]	**Control**
F. avenaceum	14.0	14.7	15.0	0	6.7	12.5	0
F. sporotrichiodes	11.3	9.0	10.3	6.3	8.7	11.5	0
F. subglutinans	14.3	9.7	8.7	7.7	7.7	12	0
F. culmorum	11.5	7.7	7.3	6.7	9.7	11.5	0
F. trincinctum	12.0	17.3	11.3	6	10.3	12	0
F. oxysporum	24.3	13	16	6.6	12.3	11	0
F. proliferatum	21	12.3	17	8.7	13	9	0
F. semitectum	19	16.7	15.7	11	10.3	19.5	0
F. scirpi	15.5	14.7	15	8.7	6.7	20	0
F. nivale	11.3	11	9.7	6.3	14.3	10	0
F. graminearum	14.5	6.3	6	6.7	12	34.5	0
F. moniliforme	16.8	6.7	6	0	7.7	13.5	0
F. solani	21.3	10.7	8.3	6	7.7	28	0

[a] Absolute concentration; [b] Nystatin Control: Pure culture with no disc

4.5 Antimould Analysis of *Tarchonanthus camphoratus*

The essential oil from *Tarchonanthus camphoratus* was found to be active against all the fungal species tested as evidenced by the zones of inhibition (Table 9 and 10). The oil showed inhibitory activity on all the 14 *Aspergillus* species tested; *A. nidulans, A. ochraceus, A. niger, A. parasiticus, A. ustus, A. sparsus, A. fumigatus, A. humicola, A. tamarii, A. flavipes, A. wentii, A. terreus, A. versicolor* and *A. flavus* (Table 9). The highest inhibition of the oil was on *A. nidulans* (14.3 mm). There was no growth of the mould in the region with a paper disc impregnated with *T. camphoratus* as shown in Appendix 2. The oil showed fairly a good antifungal activity as compared to the standard (Nystatin) that had an inhibition zone of 20 mm. The oil also had good inhibitory activity on *A. fumigatus* (12.3 mm), *A. niger* (10.7 mm), *A. ustus* (10.3 mm), *A. sparsus* and *A. tamari* (10.2 mm), *A. flavipes* (9.8 mm), *A. versicolor* (9.3), *A. wentii* (8.3 mm), *A. humicola* and *A. parasiticus* (8.3 mm), and *A. flavus* (8 mm). The least antifungal activity of the oil was observed against *A. terreus* with an inhibition zone of 6.7 mm in diameter (Table 9).

Tarchonanthus camphoratus oil also showed marked antifungal activities on *Fusarium* species as evidenced by the zones of inhibition (Table 10). The highest activity of the oil was observed against *F. oxysporum* with the largest inhibition zone of 24.3 mm as compared to 11 mm inhibition by Nystatin. This was followed by activity against *F. solani* (21.3 mm), *F. proliferatum* (21 mm), *F. semitectum* (19 mm), *F. moniliforme* (16.8 mm), *F. scirpi* (15.5 mm), *F. graminearum* (14.5 mm), *F. subglutinans* (14.3 mm), *F. avenaceum* (14 mm), *F. trincinctum* (12 mm), *F. culmorum* at 11.5 mm and lastly *F. sporotrichiodes* and *F. nivale* at 11.3 mm. The concentration of the oil was generally in the range of one times more than that of the standard antifungal (Nystatin). The oil showed more or else similar activity, across the concentration range compared with the inhibition zones exhibited by nystatin.

Essential oils are volatile substances contained in several plant organs. They play several essential functions for the plant survival including defense against invader microorganisms (Souza *et al.*, 2005). The essential oils evaluated in this study have a great variety of phytochemicals that could be considered as responsible for a higher or lower antimicrobial activity (Souza *et al.*, 2005). Phytochemicals are chemical substances characterized as organic biomolecules found and isolated from different plant derivative products such as teas, decocts, infusions, extracts and essential oils. These compounds are responsible for the biological activities exerted by several plants and their derivative products (Souza *et al.*, 2005). In general, the inhibitory action of natural products on mould cells involves cytoplasm granulation, cytoplasmic membrane rupturing and inactivation and/or synthesis inhibition of intercellular and extracellular enzymes. These actions can occur in isolate or concomitant way and culminate with the mycelium germination inhibition (Souza *et al.*, 2005).

The essential oil obtained from *T. camphoratus* is dominated by monoterpenes (80.86%) that accounts for the good antifungal activity against the fungi. Presence of an acetate moiety in the structure increases the activity of parent compound as Kiplimo (2007) points out. *Tarchonanthus camphoratus* oil has fenchyl acetate (0.20%) in minor quantity, which could have contributed to the pronounced activity of the oil. *Tarchonanthus camphoratus* contains a number of alcohols; Tenchol, z-ρ-terpineol, terpin-4-ol, ά-terpineol, 1-terpineol and ά-cadinol. The alcohols exhibit activity against micro-organisms by potentially acting as either protein denaturing agents or solvent dehydrating agents (Kiplimo, 2007). Fenchol, ά-terpineol and 1, 8-cineole are known to possess antimicrobial properties. Therefore, the essential oils of *T. camphoratus* are potential candidates to be used as antimicrobial agents against the moulds to prevent mycotoxicoses. Resistance and invasion capability of some of the mould strains used could be cited as important interfering factors to the antimould efficiency of the essential oils and phytochemicals included in the antimould assays (Souza *et al.*, 2005). Possibly, this is why the oil did not have any activity on some *Aspergillus* and *Fusarium* species tested.

4.5.1 Minimum Inhibitory Concentration of *Tarchonanthus camphoratus*

The minimum inhibitory concentration (MIC) was defined as the lowest concentration of the essential oil at which the micro-organisms do not demonstrate visible growth (Randrianarivelo *et al.*, 2009). In general, the *T. camphoratus* oil had the same or slightly lower action against all the *Aspergillus* and *Fusarium* species compared to the reference standard. The antifungal activity of the oil varied with its concentration and the kind of fungal species, indicating that its activity is proportional to its concentration. The highest activity of the oil was observed against *A. parasiticus, A. fumigatus, A. sparsus* and *A. nidulans* with an MIC of 59 mg/ml and the highest resistance was observed from *A. flavus* with an MIC of 470 mg/ml (Table 11). *Aspergillus flavus* has also been known to show resistance to basil and spearmint oils (Soliman and Badeaa, 2002). For *Fusarium*, the lowest MIC was on *F. moniliforme, F. scipri* and *F. proliferatum* with an MIC of 30 mg/ml. This was followed by activity against *F. oxysporum* with an MIC of 59 mg/ml. The highest MIC of the oil on *Fusarium* was exhibited on *F. crookwellence* and *F. sporotrichioides* with an MIC of 118 mg/ml (Table 12). Low MIC means at a very low concentration of the oil, micro-organisms demonstrate visible growth on the petri dish.

The high antifungal activity of *T. camphoratus* oil could be mainly due to the presence of the two isomers; geranial and neral, although other minor constituents like ạ-pinene have been reported to be the main cause of the antifungal activity of the oil from *Pistacia lentiscus* (*Anacardiaceae*) (Matasyoh *et al.*, 2010). The antifungal activity of the oil varied with its concentration and the kind of fungal species, indicating that its activity is proportional to its concentration. According to Matasyoh *et al.* (2007), the essential oil of *T. camphoratus*, obtained by hydro-distillation, was analyzed by gas chromatography–mass spectrometry (GC–MS) and was found to be dominated by monoterpenes, which accounted for 80.9% of the oil. The study indicated the presence of a high percentage of oxygenated monoterpenes (62.3%), of which the main constituents were fenchol (15.9%), 1, 8-cineole (14.3%) and α-terpineol (13.2%). Other monoterpenes present in fairly good amounts were α-pinene (6.87%), *trans*-pinene hydrate (6.51%), terpinen-4-ol (4.74%) and camphene (3.76%). On the other hand, b-eudesmol (5.79%) was the major oxygenated sesquiterpene present in the oil. Other sesquiterpene hydrocarbons, such as d-curcumene (2.15%), a-cadinol (1.75%) and ar-curcumene (1.69%) were also present. These could explain the good antimould activity exhibited by the oil on different fungal species. However, the inhibition level of the oil against this species in the current study was lower than that of the reference Nystatin by half indicating that, despite the resistance, the oil is relatively effective. The antimicrobial actions of monoterpenes suggest that they diffuse into and damage cell membrane structures (Sikkema *et al.*, 1995). One of the major components of this oil, 1, 8-cineole, has been known to exhibit antimicrobial activity against the bacterial strains *Escherichia coli, Salmonella typhi, Staphylococcus aureus, Rhizobium leguminosarum* and *Bacillus subtilis* (Sivropoulou *et al.*, 1997). The other major constituent of this oil, a-terpineol, has been reported to inhibit the growth of quite a number of bacteria and fungi that include *Staphylococcus aureus, Escherichia coli,* *Staphylococcus epidermidis* and *Candida albicans* (Carson and Riley, 1995; Raman *et al.*, 1995). Terpinen-4-ol, which occurs in appreciable amounts in this oil, is also reported to show activity against these organisms (Carson and Riley, 1995). It is also said to be responsible for the broad spectrum activity of the essential oil of *Melaleuea alternifolia* (tea tree oil) (Sean *et al.*, 2001). This monoterpene, isolated from *Achillea* species, showed antibacterial activity (Magiatis *et al.*, 2002). Caryophyllene oxide, although a minor constituent in the oil under study, is known to have very efficient antibacterial properties (Magiatis *et al.*, 2002). Another minor monoterpene alcohol, linalool, is reported to have a wide range of antibacterial and antifungal activity (Pattnaik *et al.*, 1997).

The findings of this study clearly show that the essential oil of *T. camphoratus* has antifungal activity and can be used in practical application in the inhibition of mould growth and mycotoxin production in stored grains. However, it should be considered that minor and major components, as well as possible interactions between the substances, could contribute to the antimicrobial properties studied. In addition, these results may justify the use of *T. camphoratus* in traditional medicine. Therefore, the essential oils from this species are potential candidates to be used as antimicrobial agents against the moulds to prevent mycotoxicoses. However, further toxicological and clinical studies are required to prove the safety of the oil as a food preservative and possibly a medicine.

Table 11: Antifungal activity and Minimum Inhibitory concentration of *Tarchonthus camphoratus* on thirteen species of *Aspergillus* after 14 days of incubation

Fungus	**Inhibition zone (mm)**									**MIC mg/ml**
	Essential oil (µg x 10^{2*})									
	94.0	**47.0**	**23.5**	**11.8**	**5.9**	**3.0**	**1.5**	**STD[a]**	**Control(-)**	
A. flavus	8.0±0.9	0	0	0	0	0	0	21.5±1.1	0	470
A. ochraceus	10.7±0.8	8.3±0.5	6.7±0.5	0	0	0	0	20±0.6	0	118
A. parasiticus	8.3±0.5	6.7±0.5	6.0±0.0	6.0±0.0	0	0	0	13±0.6	0	59
A. niger	10.7±1.7	8.3±1.1	0	0	0	0	0	28±1.1	0	235
A. tamarii	10.2±0.8	7.5±0.6	0	0	0	0	0	13±1.3	0	235
A. fumigatus	12.3±1.9	8.8±0.8	7.2±0.8	6.0±0.0	0	0	0	10.2±0.4	0	59
A. flavipes	9.8±0.8	7.3±0.5	6.0±0.0	0	0	0	0	20.2±0.6	0	118
A. ustus	10.3±0.8	6.3±0.5	0	0	0	0	0	13.5±1.1	0	235
A. nidulans	14.3±1.0	8.3±1.1	7.2±0.4	6.0±0.0	0	0	0	10.3±1.2	0	59
A. wentii	8.5±0.6	7.2±0.4	5.0±2.5	0	0	0	0	11.0±0.9	0	118
A. versicolor	9.3±1.0	8.8±0.8	7.2±0.4	0	0	0	0	19.3±1.2	0	118
A. sparsus	10.2±0.8	9.2±0.4	7.5±0.6	6.0±0.0	0	0	0	15.2±0.9	0	59
A. humicola	8.8±0.8	6.8±0.8	0	0	0	0	0	12.0±1.1	0	235

a - Nystatin (100 µg) * - The concentration values are multiplied by $\mathbf{10^2}$

Table 12: Antifungal activity and Minimum Inhibitory Concentration of *Tarchonanthus camphoratus* on eleven species of *Fusarium* after 14 days of incubation

Fungus	**Inhibition zone (mm)**									
	Essential oil (µg x 10^{2*})									
	94.0	**47.0**	**23.5**	**11.8**	**5.9**	**3.0**	**1.5**	**STD**[a]	**Control (-)**	**MIC mg/ml**
F. avenaceum	14.0±0.8	9.7±0.8	7.3±0.5	6.0±0.0	0	0	0	11.8±0.8	0	59
F. sporotrichioides	11.3±0.9	9.0±0.9	7.2±0.4	0	0	0	0	11.2±0.8	0	118
F. subglutinans	14.3±1.2	13.0±09	10.0±1.1	6.0±0.0	0	0	0	11.2±0.8	0	59
F. culmorum	11.5±1.4	10.0±0.9	7.8±0.6	6.0±0.0	0	0	0	11.3±0.8	0	59
F. oxysporum	24.3±0.5	12±1.5	10±1.1	6.0±0.0	0	0	0	12±0.7	0	59
F. proliferatum	21±1.5	13.0±1.3	9.5±0.8	7.7±0.8	6.0±0.0	0	0	9.0±0.6	0	30
F. scirpi	15.5±2.5	10.0±1.1	8.3±0.5	6.1±0.4	6.0±0.0	0	0	19.8±0.4	0	30
F. nivale	11.3±1.9	9.2±0.6	7.7±0.8	6.5±0.6	0	0	0	10.5±0.8	0	59
F. graminearum	14.5±1.2	10.3±1.5	7.0±0.9	6.0±0.0	0	0	0	35.0±0.6	0	59
F. moniliforme	16.8±2.1	13.3±1.9	8.5±0.8	7.2±0.4	6.0±0.0	0	0	13.5±1.1	0	30
F. crookwellence	10.8±1.0	9.8±0.8	7.2±0.4	0	0	0	0	20.5±0.8	0	118

a - Nystatin (100 µg)   * - The concentration values are multiplied by **10^2**

CHAPTER FIVE

CONCLUSIONS AND RECOMMENDATIONS

CONCLUSIONS

The results of this study showed that maize that appear good and clean to our eyes are also heavily contaminated with various species of mycotoxigenic moulds. Both good and mouldy maize samples from Kakamega, Trans-nzoia and Kuria districts are heavily contaminated with various species of mycotoxigenic *Aspergillus* and *Fusarium*. Communities in these areas are, therefore, slowly feeding on contaminated maize which is a serious health concern that needs to be addressed.

Most (84.4%) of the maize samples that were analyzed were positive for aflatoxins B_1 and G_1. This high contamination of good maize with aflatoxins points to an enormous exposure of humans and animals to aflatoxin poisoning and calls for immediate interventions to avert aflatoxicoses outbreaks. The essential oils of *Tarchonanthus camphoratus, Rosmarinus officinalis, Artemisia vulgare, Foeniculium vulgare* and *Piper capense* tested in this study possess varying levels of antifungal activity against *Aspergillus* and *Fusarium* species. Essential oil of *T. camphoratus* has high antifungal activity against *Aspergillus* and *Fusarium* species even at low concentrations. These oils could be used as alternatives to control mould contamination in foods and can become useful tools for application in food preservation systems.

RECOMMENDATIONS

1. There is need for improved pre and post harvest practices in preventing mould infection in maize. After harvesting, maize should be dried immediately to below 12% moisture and properly stored to prevent growth of moulds and aflatoxin production.
2. Policy makers should establish and enforce maize quality standards and regulations related to moulds and aflatoxins contamination across the region to minimize health hazards related to consumption of contaminated maize.

3. Continuous surveillance should be done to detect ear rots and storage moulds early in the field and in storage which is the key to averting their damage. This would in turn reduce the quantities of moulds and corresponding mycotoxins in maize.
4. Toxicological and clinical studies should be done to prove the safety of the essential oils as food preservatives. If proved safe, the bioactive oils should be formulated into products that small scale farmers can use to prevent mould and mycotoxin contaminations in food.
5. It would be important to establish the bioactive component/s and the mode of action of the essential oils against the moulds. Of interest also would be to determine the effect of the oils on mycotoxin production ability of the moulds.
6. The results obtained justify further screening of other essential oils for antimicrobial properties and their possible use as viable alternatives to control microbial growth and mycotoxin production in foods.

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APPENDICES

Appendix 1

Diagnostic characteristics that were used in identification of mould species according to Gilman (2001)

Aspergillus flavus: Colonies spreading rapidly, at first yellowish-green darkening with age and becoming finally brownish green. Conidiophores rough, heads vary in size, loosely radiate and becoming somewhat columnar, vesicles dome-shaped to flask-shaped. Sterigmata double. Conidia globose or somewhat pear-shaped and rough.

Aspergillus parasiticus: Colonies green, deeper in shade than *A. flavus*. Conidiophores rough and short, heads deep yellowish green. Sterigmata usually one series, conidia globose to somewhat pyriform, very rough.

Aspergillus ochraceus: Colonies ochre-coloured or buff, with submerged mycelium, conidiophores show shades of yellow in the outer layer of the wall which is rough or pitted. Heads loosely radiate and splitting , sterigmata in two series often quite large and septate.

Aspergillus niger: The hyphae are septate and hyaline more or less yellow in color. The colonies are black coloured and reverse usually colourless Conidiophores mostly arise directly from substratum and are smooth, septate or nonseptate, varying greatly in length and diameter, i.e., 200-400x7-10 and 20 μ, respectively. Conidial heads are fuscous, blackish-brown to purple-brown or in every shade to carbonous black, varying from small, almost columnar masses of a few conidial chains to the common globes or radiate heads, up to 300, 500 μ, or 1000 μ long. Vesicle globose, commonly 20-50 μ up to 100 μ in diameter. Phialides typically in two series, (biseriate), thickly covering the vesicle, primary greatly varies in length, secondary 6-10x2-3 μ. Conidia are globose, at first smooth, but later spinose with coloring substance, mostly 2.5-4 μ.

Aspergillus versicolor: Cultures show considerable range of colour. Different strains may be variously pale green, grayish green, buff or even pink in small areas, whilst a single culture may, on occasion, show patches of yellow, pink, white and green on the surface, and a deep red or plum colour (purple) on the reverse. Conidiophores smooth, colourless, vesicles globose to oval or elliptical. Sterigmata in two series.

Aspergillus nidulans: Colonies first smooth velvety and of a clear green colour, developing dirty white spots from the centre outwards as perithecia are formed, reverse deep red to purple. Conidia heads short columnar. Usually dark green with two series of sterigmata. Conidiophores smooth walled, or more or less browned; terminate in dome-like or hemispherical vesicles. Conidia globose. Perithecia usually present.

Fusarium nivale: Colonies white to pale peach to apricot with little discoloration of the agar, mycelium sparse to densely floccose. Individual hyphae irregular, conidia borne sparsely in aerial mycelium. Conidia curved, broadly falcate with a pointed apex and flattened, wedge–shaped base, 1-3 septate.

Fusarium solani: Aerial mycelium striate, sparse to dense and floccose, grayish-white. On agar typically develops a blue to bluish-brown discoloration, although occasionally a brownish pigmentation is present. Formed from lateral conidiophores, which initially may be elongated lateral phialides. Later formed conidiophores are elongated and sparsely branched. Micro conidia are broader and more oval than those of *F. oxysporum*. May have a rounded foot cell.

Fusarium poae: Aerial mycelium appears hairy to felt and assumes a powdery appearance with the formation of micro conidia. Later the aerial mycelium turns reddish brown. From below, the cultures are white, yellow, salmon to livid red or vinaceous. Formation of micro conidia begins after about 3 days. Later complex conidiophores are produced resembling a bunch of grapes. These become gradually covered in slime. The micro conidia are ampliform to globose. Macro conidia do not form readily in all cultures. They are curved falcate and slightly wider above the medium septum. They are triple septum.

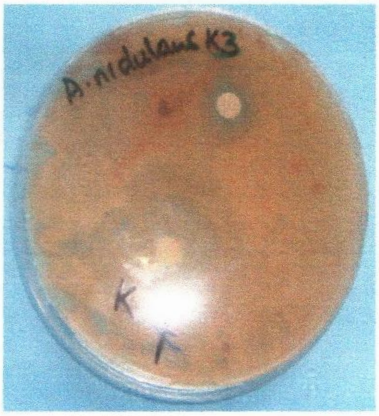
Fusarium tricinctum: Aerial mycelium sparse to floccose, white becoming carmine red to purple on the surface of the agar. Micro conidia ovate to pyriform with a minute apiculum; some later become single septate, they are formed from simple lateral conidiophores bearing 1-2 phialides, later conidiophores become profusely branched. Macro conidia are falcate, or more strongly curved and with a well marked foot cell. They are 3-5 septate.

Fusarium sporotrichiodes: Cultures generally show profuse mycelial growth on potato dextrose agar. Thin aerial mycelium is floccose, livid red below but white on the surface, then later becomes tinged with brown. Micro conidia are formed from conidiophores formed as lateral branches in the aerial mycelium. They branch once or twice and each branch terminates in 1-2 apical phialides. The phialides are cylindrical. Micro conidia are ellipsoidal to obovate becoming single septate. They are hyaline and smooth. Macro conidia develop from profusely branched conidiophores.

Fusarium moniliforme: Growth initially rather filmy, colourless and rapid. Cultures from below typically dark violet but occasionally paler, lilac, vinaceous or even cream. Aerial mycelium is generally dense, white vinaceous and often with a powdery appearance. Micro conidia are simple, lateral, subulate phialides on the aerial hyphae, micro conidia are formed in chains; they are fusiform with slightly flattened base. Macro conidia rare, when present they are non-equilaterally fusoid, delicate, thin-walled, with elongated, often sharply curved apical cell and pedicellate basal cell. They are 3-7 septate.

Fusarium oxysporum: Mycelium delicate white or peach but usually with a purple tinge, sparse to abundant then floccose, becoming felted and sometimes wrinkled in older cultures. Micro conidia born on simple phialides arising laterally on the hyphae or from short sparsely branched conidiophores. Micro conidia are generally abundant, variable, oval-ellipsoid cylindrical, straight to curved. Macro conidia, sparse, borne on more elaborately branched conidiophores. They are thin-walled, generally 3-5 septate, fusoid-subulate and pointed at both ends.

Appendix 2



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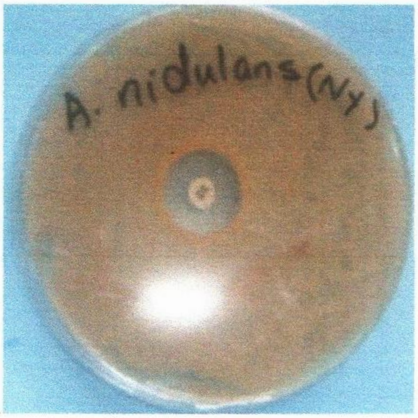

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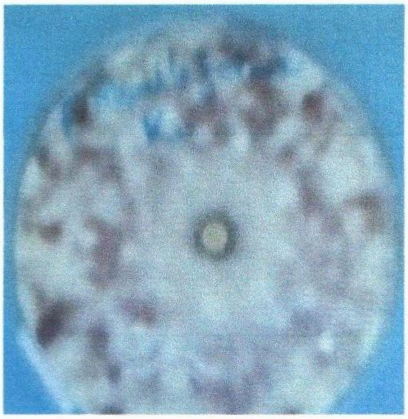

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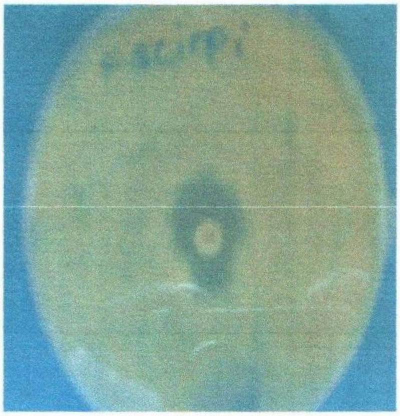

4.

Plate 1: Inhibition zones of *Tarchonanthus camphoratus* oil and *Rosmarinus officinalis* oil on *Aspergillus nidulans*

Plate 2: Inhibition zone of Nystatin on *Aspergillus nidulans*

Plate 3: Inhibition zone of *Tarchonanthus camphoratus* oil on *Fusarium moniliforme*

Plate 4: Inhibition zone of *Tarchonanthus camphoratus* oil on *Fusarium scirpi*