Aspergillus flavus AND AFLATOXIN LEVELS IN STORED MAIZE IN EASTERN KENYA AND ANTIFUNGAL ACTIVITY OF SOME PLANT EXTRACTS

By

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DECLARATION

I Theddeus Muange Kiswii declare that this thesis is my original work and has not been presented for award of a degree in any other University.

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DEDICATION

To wife Jackline Muange and my Daughters Joy Mueni, Joan Ndinda and Tracy Mwikali.

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ACRONYMS AND ABREVIATIONS

AF	Aflatoxin
AFB1	Aflatoxin B1
AFB2	Aflatoxin B2
AFG1	Aflatoxin G1
AFG2	Aflatoxin G2
ANOVA	Analysis of Variance
CBS	Central Bureau of Statistics
CDA	Czapek Dox Agar
CDC	Centre for Disease Control
CFU	Colony forming Units
CRD	Completely Randomized design
CPA	Cyclopiazonic Acid
CYA	Czapek Yeast Extract Agar
ELISA	Enzyme Linked Immunosorbent Assay
FAO	Food and Agricultural Organization
FDA	Food and Drug Administration
GY	Glucose Yeast
HBV	Hepatitis B Virus
HCC	Hepatocellular Carcinoma
KEMRI	Kenya Medical Research Institute
MEA	Malt Extract Agar
MIC	Minimum Inhibitory Concentration
MFC	Minimal Fungicidal Concentration
MRBA	Modified Rose Bengal Agar
PBS	Phosphate Buffer Solution
PDA	Potato Dextrose Agar
RPM	Rotations per Minute
SDA	Sabouraud Dextrose Agar
SDB	Sabouraud Dextrose Broth
Ppb	Parts Par Billion
WHO	World Health Organization
USP	United States Pharmacopeia

ABSTRACT

Maize is the staple crop in Eastern Province of Kenya especially in Makueni, Kangundo, Kibwezi, Machakos and Kitui South Districts. It has been noted that the abiotic and biotic stresses associated with these Districts promote growth of toxigenic fungi that produce mycotoxins in maize in storage. The aflatoxin producing species of *Aspergillus* are a common phenomenon in maize contamination that has led to frequent outbreaks of aflatoxicoses in these regions. To address this problem, this study was carried out between September 2007 and June 2008 to evaluate the incidences of *A. flavus,* determine the aflatoxin levels in stored maize from 5 Districts and evaluate the efficacy of 15 selected medicinal plants collected from Mwingi District. Four *Aspergillus* sp. that included *A. flavus, A. niger, A. ochraceous* and *A. tamarii* were isolated from maize collected from the 10 Divisions in the 5 Districts. Machakos (mean 58.80%), Kangundo (mean 58.4%), and Kitui South (mean 54.80%) Districts had significantly ($P < 0.05$) high incidences of total *Aspergillus* sp. than Makueni (mean 45%) and Kibwezi (mean 24.20%). The incidences of *A. flavus* from the 5 Districts and the 10 Divisions indicated a significant difference ($P < 0.05$). Kangundo District (mean 31.71%) had the highest incidence of *A. flavus* while Machakos District (mean 12.92%) had the lowest. Among the 10 Divisions, high incidences of *A. flavus* were found in Matungulu (mean 33.18%), Kaiti (mean 32.6%) and Kangundo (mean 30.25%) Divisions. The lowest incidence was detected in Kathiani Division (mean 10.82%). Out of the10 samples analyzed, 2 samples had > 50 ppb, 5 had > 20 ppb and the remaining 3 had < 20ppb of aflatoxin (AF) levels. The highest AF levels were recorded in samples from Kibwezi (60.35 ppb) and Kathiani (50 ppb) Divisions while the lowest was found in Kangundo (0ppb). The methanol leaf extracts of the 15 plants were evaluated for antifungal activity against *A. flavus* at different concentrations of 1000mg/ml, 750mg/ml and 400mg/ml using Agar Well Diffusion Method. Plants found to have inhibition zones of more than 10mm at 400mg/ml had their bark assayed for antifungal activity. Both the methanolic leaf and bark extracts of the fifteen plants assayed displayed concentration depended antifungal activities that were comparable to that of the reference drug Miconazole at 10mg/ml. The leaf extracts showed better antimicrobial activities than the bark extracts. For the leaf extracts, *Boscia coriacea* (mean 17.40mm) had the highest zone of inhibition followed by *Zanthoxylem chalybeum* (mean 17.20mm). For the bark extracts, *Croton megalocarpus* (mean 15.0mm) recorded significantly high antifungal activity while *Tithonia diversfolia* (mean 13.0mm) had the lowest at 400mg/ml. Both the leaf and bark extracts that were found to be effective were assayed for minimum inhibition concentrations (MIC) and minimum fungicidal concentrations (MFC) using broth (SDB) microdilution method. *Senna siamea* had the lowest MIC and MFC of 6.25mg/ml and 12.5mg/ml respectively. The preliminary phytochemical analysis of the effective plants revealed the presence of bioactive compounds that included tannins, saponins, flavonoids, terpenoids, cardiac glycosides and alkaloids. The results obtained from the study could be used as a viable management strategy against *A. flavus.*

CHAPTER 1

INTRODUCTION

1.1 Background

Maize (*Zea mays* L) is a cereal grain that was domesticated in Mesoamerica and then spread throughout the American continents and to the rest of the world after European contact with the Americas in the late 15th and early 16th centuries (The European Food Information Council, 2006). Maize is one of the most commonly grown food grain in the world. It is the main staple crop in Eastern province especially in Makueni, Machakos, Kitui South, Kangundo and Kibwezi Districts in Kenya. In these districts, the crop is planted during the long rains from March to May and also the short rains ranging from October to December. These Districts have inadequate and unreliable rains during both the rains seasons with an average seasonal rainfall in the range of 250 to 400mm. The inter-seasonal rainfall variation is large with a coefficient of variation between 45 and 58%. The temperature is in the ranges of 17 to 24^0 C (Jaetzold *et al.,* 2006). As a result the regions are often associated with abiotic stresses, such as drought, high temperatures, soil stresses, and tillage operations, as well as with biotic stresses, such as plant diseases caused by fungal pathogens, and also insect pests and weeds (Chen *et al.,* 2004). Some fungal pathogens are known to produce mycotoxins both in pre-harvest and post-harvest periods in maize.

The filamentous moulds most commonly found in stored cereal grains and oil seeds are *Aspergillus*, *Penicillium* and *Fusarium* species. These fungi can cause food spoilage, biodeterioration and are capable of producing different mycotoxins. *Aspergillu*s species are

the most common toxigenic species in various grains, legumes, oil seeds and foods and feeds (Magnoli *et al.,* 2002; Martins *et al*., 2003; Bueno *et al*., 2004). *Aspergillus flavus* Link and *Aspergillus parasiticus* Speare are the predominant fungi responsible for aflatoxin contamination of crops prior to harvest and during storage (Creppy, 2002 and Yu *et al.,* 2004). The other species of *Aspergillus* such as *A. ochraceus*, *A. niger* and *A. carbonarius* isolated from cereal and cereal-based feed produce ochratoxin A in warmer and tropical parts of the world (Accensi *et al*., 2004; Magnoli *et al*., 2006). Agricultural soil serves as the main reservoir of inocula for these fungi (Horn and Dorner, 1998). The fungus, *A. flavus* belongs to the Genus *Aspergillus*, Subdivision Deutoromycotina (Alexopoulos and Mims, 1988). The fungus grows on a wide range of agricutural commodities that include peanuts, dried corn, millet, tree nuts and cotton seeds (Michael and Ensley, 2007) and leftover foods such as rice. It is also found in water damaged carpets. This fungus is one among several species known to produce aflatoxins (Wilson and Payne, 1994).

Aflatoxins are potent toxic, carcinogenic, mutagenic, immunosuppressive agents, produced as secondary metabolites by the fungus *A. flavus* and *A. parasiticus* on a variety of food products and are the most studied and widely known mycotoxins. There are four major groups of aflatoxins: B_1, B_2, G_1 and G_2. Aflatoxin M1, a metabolite of Aflatoxin B_1 in mammals, may be found in the milk of animals eating feeds contaminated by Aflatoxin B_1 (Krishnamurthy and Shashikala, 2006; FAO, 2004). These toxins are among the most carcinogenic compounds known to cause serious problems worldwide in agricultural commodities such as maize and peanuts. They were first isolated and characterized after the death of more than 100,000 turkey poults due to the turkey X disease that was traced to the

consumption of a mold-contaminated peanut meal (Blout, 1961). Aflatoxins are associated with both toxicity and carcinogenicity in human and animal populations (Eaton and Groopman, 1994).

The diseases caused by aflatoxin consumption in man and animals are loosely called aflatoxicoses. Aflatoxicoses occur in two forms; acute aflatoxicosis that results in death and chronic aflatoxicosis that causes cancer, immune suppression, and other "slow" pathological conditions (Hsieh, 1988). The largest reported outbreak of aflatoxicosis to date occurred in western India in 1974, resulting in 397 recognized cases and 106 deaths (Krishnamachari *et.al.,* 1975). Another outbreak of acute aflatoxicosis was reported previously in Makueni District, Eastern Province of Kenya (Ngindu *et. al.,* 1982; Probst *et al.,* 2007). In April 2004, an outbreak of acute hepatotoxicity was identified among people living in Kenya's Eastern and Central provinces (CDC, 2004; Probst *et al.,* 2007). This was one of the largest and most severe outbreaks of acute aflatoxicosis documented worldwide. The outbreak resulted to 317 cases and led to 125 deaths (CDC, 2004). Of the 317 case-patients, 89% resided in four Districts (Makueni, Kitui, Machakos, and Thika). Fifty-five percent of maize products had aflatoxin levels greater than the Kenyan regulatory limit of 20 ppb, 35% had levels greater than 100 ppb, and 7% had levels greater 1,000 ppb. Maize obtained from local farms in the affected area was significantly more likely to have aflatoxin levels greater than 20 ppb compared with maize bought from other regions of Kenya or other countries (CDC, 2004). Because of the remoteness of villages in the affected Districts in Kenya and the large geographical area involved, case findings were limited to medical facilities. In addition, some people were not able to reach health-care

facilities for diagnosis and treatment. Thus the true magnitude of the outbreak was likely considerably greater than reported (CDC, 2004; Probst *et al.,* 2007).

The contamination of aflatoxins in various foodstuffs and agricultural commodities is a major problem and may vary with geographical conditions, production and storage practices, and also with the type of food. The pre-harvest practices employed in crop production such as tillage practices, fertilizer application practices, crop rotation, plant population, planting date and irrigation if not effectively managed, has a significant association of high aflatoxin levels (Campos *et al*., 2008). Delayed harvest where; insects and birds may continue to feed on maize in the field late in the season, enhances the ability of fungi to attack the kernels. This also makes the moisture content in the grain to remain high enough to allow continued development and toxin production by fungi that infect kernels. This damages grain, making it vulnerable to the toxigenic storage fungi. Ineffective management of post harvest factors such as storage moisture, temperature, aeration, insect activity and sanitation of storage facilities enhance mycotoxin problems in dried grain (Campos *et al.,* 2008).

Detoxification by many control measures, such as chemical, biological, and physical means, have been tried to reduce or eliminate aflatoxin contamination in maize, but none appears to be economically feasible (Lillehoj and Wall, 1987). Fungicides are the primary means of controlling postharvest diseases. However, repeated use of certain chemical fungicides in maize storage structures has led to the appearance of fungicide-resistant populations of storage pathogens (Feng, and Zheng, 2006). In recent years there has been considerable pressure by consumers to reduce or eliminate chemical fungicides in foods.

Further, the use of synthetic chemicals to control postharvest diseases has been restricted due to their carcinogenicity, teratogenicity, high and acute residual toxicity, long degradation period, environmental pollution and their adverse effects on food and effects human health (Unnikrishnan and Nath, 2002). Natural plant extracts may provide an alternative way to protect foods or feeds from fungal contamination (Yin and Cheng, 1998). They provide a source of inspiration of novel drug compounds, as plant derived medicines have made large contributions to human health and well- being (Lewis and Elvin - lewis, 1995).

Despite the vast literature on the efficacy of plant material in controlling mycotoxigenic moulds, there have not been adquate efforts of large-scale trial of these plants on the farmers' field (Bankhole and Adebanjo, 2003). This study was therefore aimed at evaluating the efficacies of extracts of 15 selected medicinal plants that are locally available against the growth of *A. flavus* and aflatoxin production. The plants used in this study were selected based on the traditional medicinal use and experience passed from generation to generation, virtually by word of mouth. The plants have been used for a long time for treating various infections of bacterial, protozoan and fungal origin, cleaning and disinfection of the storage structures, and for the storage of varied grains. All these plants were collected from Mwingi District in Kenya. The frequent aflatoxicoses outbreak in Kenya's Eastern Province has been attributed to high levels of aflatoxins caused by high incidences of *A. flavus* (CDC, 2004). The study was also aimed at determining the incidences of the *A. flavus* infection and aflatoxin levels in stored maize in the 5 Districts.

1.2 Statement of the Problem

Transitory food shortages in Kenya's Eastern Province especially Makueni, Machakos, Kitui South, Kangundo and Kibwezi Districts, force hundreds of families to eat grains contaminated with aflatoxin producing molds that grow as a result of improper pre-harvest, harvest and post harvest practices. Potentially high incidences of the fungi in the *Aspergillus* species have caused food spoilage, biodeterioration and are capable of producing different mycotoxins that include aflatoxins. Consumption of food contaminated with aflatoxins has caused both chronic and acute aflatoxicosis that has resulted in loss of livestock and human life. The presence of fungal infection of maize in the field usually leads to lower yields for food and fibre crops, nutrient losses, alteration of organoleptic properties and diminishment of product shelf life in the market leading to adverse economic effects. Further, the use of synthetic chemicals to control storage fungi particulary *Aspergillus* species in stored maize has been restricted due to their carcinogenicity, teratogenicity, high and acute residual toxicity, long degradation period, environmental pollution and their adverse effects on food and side effects on human human health. This therefore prompted for the study to test the efficacy of selected extracts of plants collected from Mwingi District that may later be used as alternative manangement strategy in the control of *A. flavus* and aflatoxin production in stored maize.

1.3 Objectives

1.3.1 General objectives

To evaluate the incidence of *A. flavus* and aflatoxin levels in stored maize in Eastern province of Kenya and identify management strategies of the fungus.

1.3.2 Specific objectives

(i) To determine the incidence of *A. flavus* in stored maize from Eastern Kenya.

(ii) To determine the aflatoxin levels in stored maize from Eastern Kenya.

(iii) To evaluate the efficacy of selected plant extracts on the growth of *A. flavus.*

(iv) To identify the bioactive agents present in the selected plant extracts.

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1.4 Research hypotheses

(i) The incidence of *A. flavus* in stored maize in Eastern Kenya does not have an effect on aflatoxin contamination.

(ii) The aflatoxins levels in stored maize from Eastern Kenya do not have an effect on maize contamination.

(iii) The extracts from the selected plants have no effect on the growth of *A. flavus* .

(iv) The selected medicinal plants have no bioactive agents that may inhibit *A. flavus.*

1.5 Justification and Significance of the study

The severe aflatoxicoses that have occurred in Eastern Kenya has caused great loss of human life and livestock. The high incidences of *A. flavus* in stored maize have caused aflatoxin poisoning in this region. This then calls for assessment of the occurrence of the

fungi and aflatoxin levels in stored maize as the first step in the prevention and management of aflatoxicoses. The Eastern Province of Kenya has not received appropriate attention in pursuit of the level of incidences of *A. flavus* and its link to aflatoxin contamination. Therefore, data generated from the study will give an insight into the causes of aflatoxicoses outbreaks in this region. It will also provide adequate information on the levels of aflatoxin in the maize samples. The results will be compared to the recommended limit set by the Food and Agricultural Organization (FAO). The results from the tested medicinal plants will lead to identification of plant extracts with high efficacy that could provide a viable management strategy against *A. flavus* and other postharvest diseases.

CHAPTER 2

LITERATURE REVIEW

2.1 Aflatoxins

Aflatoxins are potent toxic, carcinogenic, mutagenic, immunosuppressive and teratogenic agents produced as secondary metabolites by *A. flavus* and *A. parasiticus* (Krishnamurthy and Shashikala, 2006). These toxins are named after the fungus producing them, e.g. "A" from the genus name *Aspergillus*, "fla" from the species name *flavus* added to toxin to give the name aflatoxin. There are 18 different types of toxins in the aflatoxin group identified. Among these aflatoxin toxins, the major members are aflatoxins B_1, B_2, G_1, G_2, M_1 and M_2 (Wrather, 2008). Aflatoxin B_1 is produced most abundantly and is the most toxic followed by G_1, B_2 and G_2. Aflatoxins B_1, B_2, G_1 and G_2 are classified as Group I human carcinogens whereas M_1 is classified as Group 2B probable human carcinogen (Krishnamurthy and Shashikala, 2006).

Aflatoxin is extremely durable under most conditions of storage, handling and processing of seeds or in foods or feeds made from contaminated seeds. It is very heat stable and will withstand temperatures up to boiling. Aflatoxin levels in maize may decline in storage, but may still be present after 7 years (Abbas, 2005). Aflatoxin becomes more prevalent, and therefore more of a food safety concern, during a drought because low rainfall and high temperatures encourage the growth and survival of the molds that produce the toxins. Crops stressed by drought and high temperatures and/or weakened by insect or other damage, are more susceptible to mold growth and subsequent aflatoxin contamination. The aflatoxin-

producing molds can grow on crops in the field, poorly dried harvested crops in storage and processed food and feed products (Abbas, 2005).

2.2 Effects of Aflatoxins on Plants, Animals and Humans

Aflatoxins are of economic and health importance because of their ability to contaminate human food and animal feeds, in particular cereals, nuts and oilseeds (Kaaya and Warren, 2005). The toxins have adverse effects on plants, animals and humans. They are responsible for damaging up to 25% of the world's food crops, resulting in large economic losses in developed countries and human and animal disease in under-developed countries (Abbas, 2005).

2.2.1 Effects of aflatoxins on plants

A. flavus grain mold on maize is often characterized by visible light as green mold on the surface of the kernels. This surface mold can develop anywhere on the ear, but is most often observed at the base of the ear. Visible mold growth is not always evident on colonized kernels and not all colonized kernels will be contaminated with aflatoxin. However, colonized kernels with no visible mold may contain aflatoxin. The Food and Agricultural Organisation (FAO) estimates that 25% of the world's crops are affected by mycotoxins, of which the most notorious are aflatoxins. These leads to adverse economic effects that include lower yields for food and fibre crops (Sétamou *et al.*, 1997).

2.2.2 Effects of Aflatoxins on animals and humans

The disease caused by aflatoxin is called aflatoxicosis. Aflatoxicosis is neither infectious nor communicable; it cannot be spread from one animal to another or from one human to another. There is the acute aflatoxicosis that results in death and chronic aflatoxicosis that results in cancer, immune suppression, and other "slow" pathological conditions. The livestock and poultry producers suffer from aflatoxin losses that include death and more subtle effects of immune system suppression, reduced growth rates, and losses in feed efficiency due to consumption of aflatoxin-contaminated feeds (Stack and Carlson, 2006). The primary target of aflatoxin is the liver. Depending on the duration of feeding on contaminated grain or food products and the amount of aflatoxin ingested, the liver may fail to function or liver cancer may develop. Recovery from liver failure depends on the extent of damage. If damage is not too extensive, full recovery can be expected if the contaminated feed or food products are removed from the diet.

In addition to the production of aflatoxin, *Aspergillus* species of mold can affect humans or animals in two other ways. Some people and animals are allergic to *Aspergillus* species and exhibit either acute or chronic reactions to the mold itself. *Aspergillus* molds can infect animals, including humans, with inadequate immune system function causing a disease called aspergillosis. It is an invasive disease of the lungs, although colonization of other organs can occur. Aspergillosis is a serious disease that is often fatal. Dust masks or respirators should be worn by grain handlers to minimize exposure to these fungi and to aflatoxin contaminated dust (Stack and Carlson, 2006).

The other effects of aflatoxins are of economic and health importance because of their ability to contaminate human food and animal feeds, in particular cereals, nuts and oilseeds (Arim, 1995). The economic impact of aflatoxins is derived directly from crop and livestock losses due to flatoxins and directly from the cost of regulatory programs designed to reduce risks to human and animal health. Nevertheless, aflatoxins reputation as a potent poison may explain why it has been adopted for use in bioterrorism (Bennett and Klich, 2004).

2.3 Aflatoxin contamination and prevention

Exposure to aflatoxins occurs primarily through ingestion of contaminated foods (Fung and Clark, 2004) and can cause hepatic and gastrointestinal injury and have immunosuppressive, teratogenic, and oncogenic effects. Chronic low-level aflatoxin exposure can increase the risk for hepatocellular carcinoma (Peraica *et al.,* 1999). Severe, acute liver injury with high morbidity and mortality has been associated with high-dose exposures to aflatoxins (Chao e*t al.,* 1991). Ingestion of 2-6 mg/day of aflatoxin for a month can cause acute hepatitis and death (Patten*,* 1981).

Knowing the hazards of aflatoxin exposure, the need for protection of foods and feedstuffs against aflatoxin is universally recognized and several approaches have been suggested. The US Food and Drug Administration (FDA) have established acceptance level of 20 ppb for aflatoxin in maize for human consumption, with the level in milk being even lower (0.5 ppb) (Grybauskas *et al.,* 2000). Maize containing aflatoxin levels of 20 ppb or more should thus not be consumed by humans, young poultry and swine (Grybauskas *et al.,* 2000). The

maize grains with contamination levels of 200 ppb and 300 ppb may be fed to finishing swine and cattle, respectively (Munkvold, 2003). However different countries have different accepted levels for aflatoxins in foods**.** In some European countries aflatoxin levels are regulated below 5 ppb (Grybauskas *et al.,* 2000) while in the United States, aflatoxin concentrations are limited to 20 parts per billion (ppb), a level also adopted by Kenyan authorities. The 2004 outbreak resulted from widespread aflatoxin contamination of locally grown maize, which occurred during storage of the maize under damp conditions. Efforts should focus on the prevention of aflatoxin exposure by implementing extensive food replacement, without which, the epidemic can be expected to continue. Longer-term requirements include strengthened surveillance; increased food inspections to ensure food safety; and local education and assistance to ensure that maize is harvested correctly, dried completely, and stored properly.

2.4 Management options to minimize aflatoxin contamination

2.4.1 Management practice to minimize aflatoxin problems in maize fields

The following are the management practices that help reduce aflatoxin problems in maize fields according to Munkvold (2003) and Wrather (2008).

1. Plant regionally adapted hybrids.
2. Use a balanced fertility program designed for optimum yields.
3. Select planting dates appropriate for your area.
4. Follow recommended management practices to limit damage by ear feeding insects.
5. Attempt to best utilize your irrigation practices to deliver optimum water from silking stage to late dough stage.

6. Make adjustments in combine ground speed and cylinder speed to minimize trash and broken kernels in hopper. Aflatoxin is often associated with broken or lightweight kernels.
7. If drought has occurred during the season, consider harvesting irrigated or high yielding fields separately from dryland or poor yielding fields.
8. Begin maize harvest when grain moisture is about 24% and dry the grain to 15% moisture within 24 hours or as soon as possible.
9. Maize which collects in auger wells and pits around dump stations frequently contains the aflatoxigenic molds or aflatoxins. Thoroughly clean all such areas before and after use. Remove leftover grain from trucks, trailers, holding bins, drying facilities and storage structures before beginning a new lot of grain.

2.4.2 Management practices to minimize aflatoxin problems in stored maize

To minimize the risk of *Aspergillus* grain mold and aflatoxin contamination in maize during storage the following are the management practices that should be undertaken (Stack and Carlson, 2006; Wrather, 2008).

1. Thoroughly clean storage structures, areas around them and all grain handling equipment before putting any grain in storage. This is done each season to remove old grain and residue. Grain mold fungi survive for long periods in storage structures. The structures can be sanitized using a bleach solution (6 ounces of bleach per gallon of water). Storage structures with false floors may require additional effort to remove

fines and broken kernels. Sanitation in storage structures is important especially when the ability to maintain cool and dry storage conditions is lacking.

2. Sort maize grain going into storage to remove lightweight and broken kernels as well as foreign material and fines.
3. Moisture content is by far the most important factor affecting the growth of microorganims in stored maize. After harvest, maize should be dried to 15% moisture content within 24 hours. Grain going into long term storage should be dried to 13% moisture.
4. Check stored grain on regular basis and aerate as needed to maintain low moisture and proper temperature. This aerates the grain to safe and equalized temperatures through the grain mass. Good air circulation throughout the storage structure is important.
5. Protect grain from insects to minimize insect damage during the growing season and during postharvest storage.
6. Minimize mechanical damage during harvest and postharvest handling.
7. Monitor storage facilities regularly to detect grain mold development. This requires a systematic sampling plan to account for the unique design characteristics of each storage facility.
8. Have grain analyzed for aflatoxin content. Proper protocols should be used to collect samples and have them shipped to a lab for analysis.

2.4.3 Management options to minimize aflatoxin using chemicals

Fungicides are used for the management of *Aspergillus* grain mold and from aflatoxin contamination. Included are Fludioxonil, strobilurin, berberine hemisulfate, and phenolic

agents, vanillin, vanillylacetone, cinnamic acid, *m*-coumaric acid, and veratraldehyde. (Ojeda-Contreras *et al.,* 2008).

2.4.4 Management options to minimize aflatoxin using host plant resistance

Although maize hybrids are reported to vary in susceptibility to *A. flavus*, susceptibility to grain mold and aflatoxin contamination are not characteristics listed in the seed catalogs. The resistance in some of these sources has been linked to one or more kernel proteins that inhibit either fungal growth or aflatoxin production. Currently, maize hybrids with improved resistance to *A. flavus* and aflatoxins are being used, but the level of resistance is not yet adequate to prevent unacceptable aflatoxin concentrations in some fields. Active breeding programs are under way in the public sector in persuit of resistant maize hybrids that may lead to low aflatoxin contamination (Munkvold, 2003).

2.4.5 Management options to minimize aflatoxin using cultural means

This includes pre-harvest, harvesting and post-harvest practices. During pre-harvest; the general strategy is to alter the conditions under which the crop is grown so that infections by the offending fungus or fungi are avoided. Tactics employed in this struggle include those used to battle most plant diseases: tillage practices, fertilizer application practices, crop rotation, plant population, planting date, and irrigation. Cultural practices that tend to expose plants to greater drought stress will lead to higher levels of aflatoxins (Munkvold, 2003). For harvesting and drying: Management of mycotoxins requires late-season scouting in order to make informed decisions about harvest timing, postharvest grain handling, storage, and marketing. Timing of harvest can have major consequences for the ultimate

level of mycotoxin accumulation. In postharvest: Grain storage practices can be altered to decrease the likelihood of postharvest mycotoxin development.

2.5 Taxonomic description

The fungi *A. flavus* and *A. parasiticus* grow rapidly on standard identification media such as Malt Extract agar (MEA) or Czapek Yeast Extract agar (CYA) (Klich , 2002), and produce yellow green conidia on colonies which are otherwise uncoloured (Klich 2002). *A. flavus* is a yellow - green mold found on ears of corn in the field or kernels in storage. The hyphae are septate and hyaline. Conidial heads are radiate to loosely columnar with age. Conidiophores are coarsely roughened, uncolored, up to 800 µm long x 15 - 20 µm wide, vesicles globose to subglobose (20 - 45 µm), metulae (8 - 10 x 5 - 7 µm) covering nearly the entire vesicle in biseriate species. Some isolates may remain uniseriate, producing only phialides (8 - 12 x 3 - 4 µm) covering the vesicle. Conidia are smooth to very finely roughened, globose to subglobose, 3 - 6 µm in diameter (Sutton *et al.,* 1998; Klich, 2002). *A. flavus* produces AFB1 and AFB2 while *A. parasiticus* produces AFB1, AFB2, AFG1 and AFG2 (Bennett and Klich, 2004). *A. flavus* isolates usually make only B aflatoxins and less than 50% of isolates are toxigenic, while *A. parasiticus* isolates produces G as well as B aflatoxins, and are invariably toxigenic (Krishnamurthy and Shashikala, 2006).

The aflatoxin producers in *A. flavus* can be classified as L or S strains according to the size of the sclerotia. The S strains produce numerous small sclerotia that are less than 400 µm and fewer conidia while the L strains produce fewer, larger sclerotia that are greater than

400 µm (Bennett and Klich, 2004). The S strains produce relatively high levels of aflatoxins, while L strains produce low levels of aflatoxins, or are atoxigenic (Bennett and Klich, 2004). *A. flavus* also produce sterigmatocystin. There are chemical structural similarities between AFB_1, AFG_1, and sterigmatocystin (Guengerich *et al*., 1991). Sterigmatocystin is eventually converted to AFB_1 (Brown *et al*., 1998).

2.6 Distribution of *A. flavus* in nature and in foods

Agricultural soil serves as the main reservoir of inocula for this fungus (Scheidegger and Payne, 2003). The fungus can grow and release aflatoxins in several foods and feed compounds (Michael and Ensley, 2007). Peanuts, dried maize, millet and leftover foods such as rice are significant substrates of *A. flavus*. Cereals are a common substrate for growth of *A. flavus* but, unlike the case of nuts such as groundnuts and oilseeds, small grain cereal spoilage by *A. flavus* is almost always the result of poor handling (Michael and Ensley, 2007).

2.7 Transmission and life cycle *A. flavus*

The fungus grows saprophytically in infected plant tissues such as maize kernels, cobs, and leaf tissue that remain in the soil and contribute to primary inoculum (Scheidegger and Payne, 2003). The inoculum for *A. flavus* is spread through water, wind and also transmitted through insects and bird damage. The fungus produces prodigious numbers of airborne conidia on sporogenic sclerotia that are disseminated by air currents and possibly by insects. *A. flavus* infects maize seeds through wounds (Abbas, 2005).

2.8 Factors favouring infection by *A. flavus* and aflatoxin production

2.8.1 Factors in the field

Plant stress favours colonization of maize kernels and infection by *A. flavus*. Contamination is influenced by many factors and can occur at any stage of food production, from pre-harvest to storage (Campos *et al*., 2008) and processing. High aflatoxin levels are often associated with abiotic stresses, such as water (drought), temperature (heat), soil stresses (nitrogen deficiency), agronomic (cropping pattern, variety, planting date, delayed drying and storage conditions) and tillage operations. It is also associated with biotic stresses, such as insects and weeds (Chen *et al.,* 2004). All of these conditions weaken the host or provide a means of entry to the spores to establish a foothold in/on the host.

2.8.1.1 Abiotic stresses

Fungal growth may begin on maize at moisture content lower than 18.0 %. The fungus *A. flavus* grows best on maize at 18.0-18.5 % moisture level (Wrather, 2008). Then as the fungus grows, respiration occur releasing heat and moisture into the surrounding environment in the grain mass. This results in an increase in the moisture content and temperature of the surrounding maize, causing a hot spot. If moisture content and temperature continue to rise, the environment for *A. flavus* becomes more favorable. At 20% moisture content and above, other fungi grow better and crowd out *A. flavus* (Wrather, 2008). Aflatoxin contamination has been reported to be greater in years with below average rainfall (Payne, 1992). Drought stress can lead to cracks in maize kernel surfaces, providing additional entry sites for hyphae of *A. flavus* (Wrather, 2008). There is strong

evidence for drought stress alone to be a contributor to elevated aflatoxin levels. According to Abbas (2005), moisture levels in maize below 12 to 13% inhibit growth of the fungus at any temperature.

The most serious aflatoxin problems have occurred in years with above average temperature and below average rainfall. The first report confirming the incidence of aflatoxin found in pre-harvest grain also pointed to a higher incidence of aflatoxin contamination of maize grown in warmer regions of the USA (Anderson *et al*., 1975). Temperatures conducive to growth of the fungi are 17 - 42°C with aflatoxin production between 25 - 35°C. At the same time, temperatures above 30°C can start to cause heat stress in maize plants, thus leaving the invading fungus at an even greater advantage (Chen *et al.,* 2004). Payne *et al*. (1992) also found increased kernel infection at higher temperatures and pointed out that *A. flavus* might have an increased parasitic ability at higher temperatures. The importance of temperature and humidity was corroborated by Widstrom *et al.* (1990), suggesting that, contrary to the standard recommendations for early planting, such plantings in the deep South U.S.A were at a higher risk of aflatoxin contamination compared with plantings aimed at changing the time period for grain-filling.

Plant stress is said to occur when nutrients become so limited that yield is reduced. According to Payne *et al.* (1992) kernels from plants receiving no nitrogen undergo nitrogen stress and produce more aflatoxin than those from plants receiving an optimum nitrogen dose. Jones, (1987) suggested that mineralization of nitrogen on highly organic soils, compared with sandy soils, tended to reduce aflatoxin contamination. Reduced

availability of nitrogen also occurs with drought stress, which compounds the effects of low moisture alone.

2.8.1.2 Biotic stresses

The role of insects in the infection and contamination processes has been reviewed extensively (Barry, 1987). Insects physically move conidia adhering to their bodies to plant parts in feeding and leave them via defecation (Diener *et al.,* 1987). These insects include the European corn borer (*Ostrinia nubilalis* Hubner), corn earworm (*Helicoverpa zea* Boddie) or fall armyworm (*Spodoptera frugiperda* Smith). The maize weevil (*Sitophilus zeamais* Motschulsky) is of special interest with respect to the aflatoxin contamination problem because it functions as both a pre-harvest and storage insect. Heat and moisture generated by weevil activity in stored maize grain has been shown to enhance growth of *A. flavus* (Dix and All, 1987). Another vector of the fungus is sap beetle (Nitidulidae). (Lussenhop and Wicklow, 1990). These insects through feeding cause injuries to ears thus provide sites for fungal infection. Resistance to insect feeding should be effective in reducing fungal infection and aflatoxin accumulation in the grain (Williams *et al.,* 1997).

Weed competition also contributes to stress-induced aflatoxin problems. Seasons with above average rainfall during the early crop development followed by drought during the reproductive stages encourage weed competition. According to Jones (1987), these would likely be most evident in sandy soils that normally have limited moisture-holding capacities. Eliminating weeds obviously reduces water usage and assists in preventing water stress on the crop and yield losses in dry-land production systems. An investigation that compared three cultivation rates to control of weeds found no significant differences

among the treatments for aflatoxin production in the pre-harvest crop (Bilgrami *et al*., 1992), but it did not imply that weed control was not important.

2.8.2 Factors in storage

The extent and severity of both invasion by *A. flavus* and the production of aflatoxin in the stored grain are influenced by several factors including moisture content and temperature of stored grain, condition of grain going into storage and length of storage. According to Wrather (2008), *A. flavus* can also develop or continue to develop on maize in storage.

Moisture level of the stored food products that the fungus inhabits determines the amount of aflatoxin produced. In stored maize grain, *A. flavus* requires at least 17.5 % grain moisture for growth (Abbas, 2005). Drying maize grain, after harvest, to approximately 13 % moisture is one of the most important methods to prevent aflatoxin contamination during storage (Abbas, 2005).

The fungus *A. flavus* grows best at high temperatures. The range of temperatures conducive to growth of the fungi is 17 - 42°C with aflatoxin production between 25 - 35°C.

Maize contaminated with *A. flavus* going into storage will deteriorate at lower moisture content, at a lower temperature and in a shorter time than grain that is free or almost free of *A. flavus* as it goes into storage. Maize kernels with cracks or breaks in the pericarps or

seed coats, broken kernels or other physical damage is more subject to invasion by *A. flavus* (Wrather, 2008).

It is important to note that the presence of *A. flavus* in maize does not necessarily mean that aflatoxin is also present in that maize. Circumstances that favor mold growth may also favor mycotoxin production but mold growth may also occur with little or no mycotoxin production.

2.9 Control of *A. flavus* and aflatoxin production using medicinal plants

The widespread use of synthetic pesticides have significant drawbacks including increased cost, handling hazards, concern about pesticide residues on grains, and threat to human health and environment (Phongpaichit *et al.,* 2005). Further, the use of synthetic chemicals to control postharvest diseases has been restricted due to their carcinogenicity, teratogenicity, high and acute residual toxicity, long degradation period, environmental pollution and their adverse effects on food and side effects on humans (Unnikrishnan and Nath, 2002). The side effects of synthetic fungicides in maize storage mean that alternative strategies need to be developed for reducing losses due to postharvest decay that have caused negligible risk to human health and environment (Phongpaichit *et al.,* 2005).

Several nonchemical treatments have been proposed for fungal decay control such as microbial antagonists, plant extracts and essential oils. Public awareness of these risks has increased interest in finding safer insecticides or alternative stored product protectants to replace synthetic chemical pesticides. One such alternative is the use of natural plant

protectants that have fungicidal activity, because they tend to have low mammalian toxicity, less environmental effects and wide public acceptance (Hamilton-Kemp *et al.,* 2000). Medicinal plants have been used for centuries as remedies for human diseases because they contain components of therapeutic value. Recently, the acceptance of traditional medicine as an alternative form of health care and the development of microbial resistance to the available antibiotics has led authors to investigate the antimicrobial activity of medicinal plants (Nostro *et al*., 2000). In recent years, a number of studies have been conducted in different countries to prove such efficiency (Unland and Higgins 2006; Fontenelle *et al.,* 2007; Matasyoh *et al.,* 2007). The use of plant extracts and phytochemicals with known antifungal properties can be of great significance in the management strategies against storage fungi in maize. According to Nascimento *et al.* (2000), antimicrobial properties of plants are due to compounds synthesized in their secondary metabolism.

Natural compounds from plants have been used traditionally to preserve foods in countries like Japan, Russia and India (Wilson *et. al.,* 1992). These compounds are becoming popular as a source of safer and more effective substances that produce antimicrobial agents. The extracts and powders of some local plants show the ability to suppress growth of toxigenic fungi and hence toxin production in synthetic media (Leite *et al.,* 2006). The extracts of several wild and medicinal plants have been tested against aflatoxin-producing fungi (Bilgrami *et al.,* 1980). Numerous studies have documented the antifungal properties of plant essential oils (Sokmen *et al*., 2004) that include oils from *Plectranthus elegans* to inhibit the germination of the fungus *Cladosporium cucumerinum* (Marwah *et al.,* 2007).

It is evident that studies of the fungicidal and fungistatic activity of different natural phytochemicals have been focused on controlling growth and aflatoxin synthesis by *Aspergillus* section *Flavi*. Caffeic and vanillic acids have been shown to inhibit the growth of *A. flavus* and *A. parasiticus* at 0.2 mg/kg (Aziz *et al*., 1998; Ojeda-Contreras *et al.,* 2008). Extract from *Garcinia pedunculata*, with a high content of phenolic acids, inhibited the growth and aflatoxin production in *A. parasiticus* (Adegoke *et al*., 1996) and *A. flavus.* According to Joseph *et al*. (2005) the extract from *Garcinia*, have a rich source of phenolic acids that inhibited more aflatoxin production than growth of *Aspegillus* section *Flavi.* Eugenol, a phenol compound, inhibited aflatoxin production without any significant effect on growth of the organism. It has been shown that utilization of antifungal compounds derived from plants like isothiocyantes (Troncoso-Rojas *et al*., 2005), plant volatile compounds (Neri *et al*., 2006), hexapeptides (Lopez-Garcia *et al.*, 2003), essential oils (Fraternale *et al.*, 2004), phenolic compounds (Ahn *et al.*, 2005), polyphenols like *trans*-resveratrol (Urena *et al*., 2003) and antifungal compounds synthesized by bacteria like phenazines (Kumar *et al.*, 2005) have been used to control fungi. Some of the natural products, such as extracts from *Maesa lanceolata* Forsskal (Okemo *et al.,* 2003), some spices (Hasan and Mahmoud, 1993), phenols, and many essential oils (Ramkumar *et al.,* 2007), have been reported as effective inhibitors of fungal growth and aflatoxin production. The antifungal properties are caused by many active phytochemicals, including flavonoids, terpenoids, carotenoids, coumarins and curcumines (Tepe *et al.,* 2005).

Although hundreds of plant species have been tested for antimicrobial properties (Arora and Ohlan, 1997), the vast majority have not yet been adequately evaluated (Balandrin *et al.,* 1985). Therefore the objective of the current study was to investigate the fungicidal and

the anti-aflatoxigenic effects of the selected medicinal plant extracts against *A. flavus* with the aim of developing cost effective and environmental friendly treatment system to control pests on stored maize.

CHAPTER 3

MATERIALS AND METHODS

3.1 Study site

The study area consisted of Makueni, Machakos, Kitui south, Kangundo and Kibwezi Districts that make part of Eastern province of Kenya (Figure 3.1). These regions make part of the Ukambani region that is situated on a predominantly semi-arid, eastward-facing slope, which becomes progressively lower and drier to the east. It is part of Kenya's Eastern Foreland Plateau, an eroded basement complex broken by residual hill masses and occasionally overlain by Tertiary volcanics (Bernard *et al.,* 1989; Jaetzold *et al.,* 2006). This part of Kenya forms an environmental gradient of decreasing altitude (from 2,100 m to 440 m), increasing temperatures, and decreasing moisture (from 1, 270 to 381 mm average annual rainfall) from west to east (Jaetzold *et al.,* 2006). Rainfall, except in the hill regions, is low and unreliable. The precipitation pattern is bimodal, with long rains falling between March and May and short rains from October to December (Jaetzold *et al.,* 2006). The combined area of the four districts is approximately 45,000 km², although one-fifth of Kitui, some 6,300 km², lies within Tsavo National Park and is therefore unavailable for use by the Akamba.

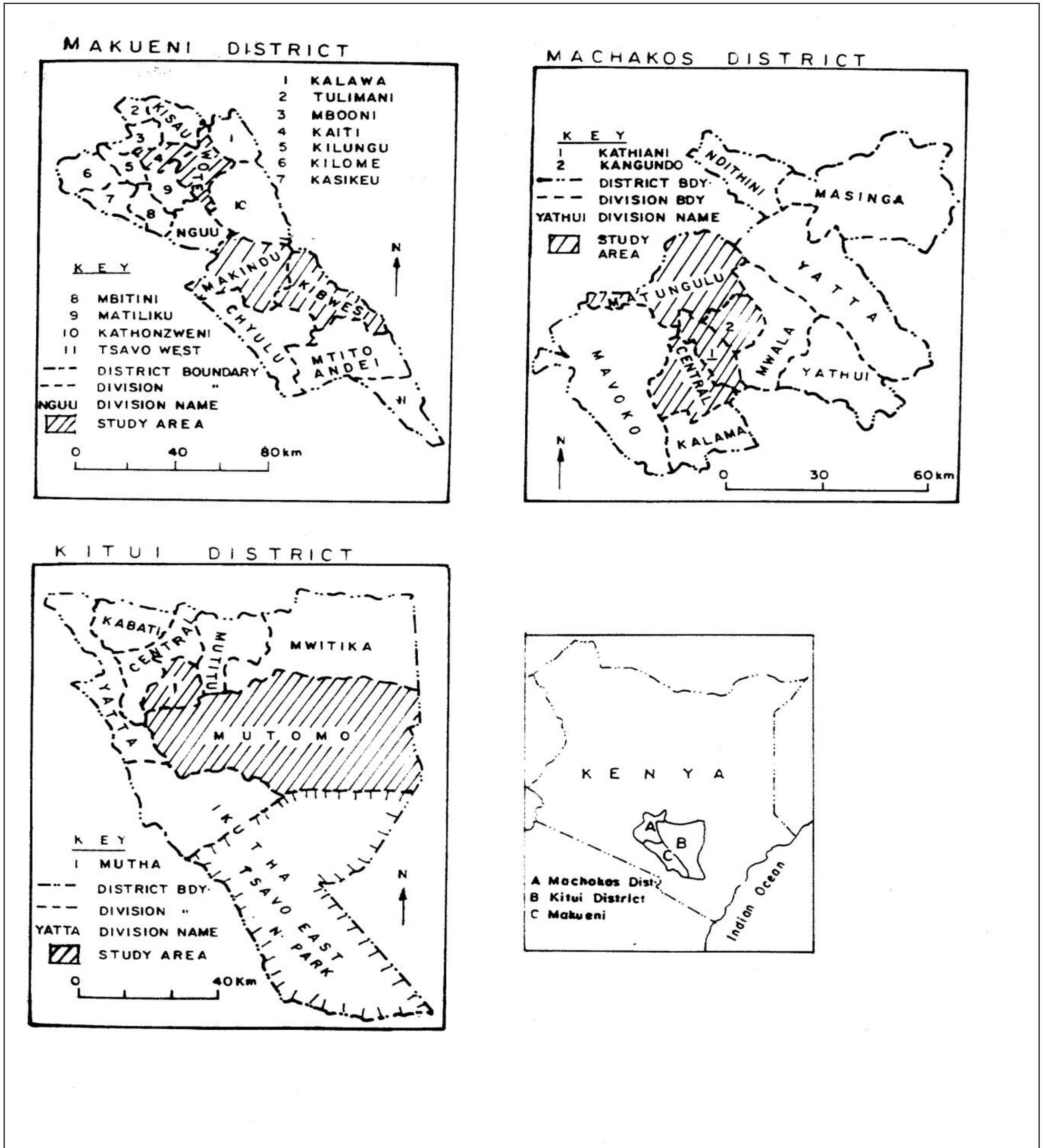
More than 95 per cent of the four Districts population is Akamba (Jaetzold *et al.,* 2006), and approximately 85 per cent of all Kamba speakers reside in Ukambani. The estimated total population of these five istricts is 2.8 million (Central Bureau of Statistics, 1999; Jaetzold *et al.,* 2006). Overall, the area has a rural population that is primarily from the Akamba ethnic group. Most of the local population engages in small-scale, mixed farming

that includes some livestock. Maize is the primary dietary staple and the main crop produced. At harvest, farmers store most of their maize for household consumption in varied storage structures and sell the rest to meet other household needs.

3.2 Maize sample collection

Maize samples were collected from Kangundo, Machakos, Makueni, Kibwezi and Kitui South Districts in Eastern Kenya. From each District, two Divisions were selected and the maize samples collected from ten farmers randomly selected from each Division. Samples of 1kg were collected from maize stored by the farmers in the various storage structures that included galleries, houserooms, bags, large gourds and temporally wooden structures for one month or more. Some of the storage structures used by farmers for maize storage in this region are shown in plates 3.1a - 3.1f. The samples were collected from 90kg bags or any other storage structure with multiple sampling areas from the same bag or structure using the respective farmers' tools. Several bags/structures from the same farmer in the same store produced samples that were combined to make a sample of 1 kg sample. These were then packed and carried in paper bags and later the samples from the ten farmers in each Division were mixed to make a bulk sample of 10 kg. The total number of samples collected was 100. The samples were taken to the laboratory; their moisture content determined using a moisture meter (Model GMK-3O3, G-won Hitech Co., South Korea) and dried to moisture level of 13%. The samples were then divided into two; one third of the sample for mycological analysis and two-thirds for aflatoxin analysis packed and stored in paper bags at 4ºC in the laboratory.

Figure 3.1: Maps showing the study areas



NB; The shaded regions in the maps represent the 10 Divisions in the study area. The 5 Districts in the study were created from the 3 old Districts; Makueni, Machakos and Kitui Districts shon in the maps above.

3.3 Determination of incidences of *Aspergillus* species in stored maize from Eastern Provincce

A sample of 100g maize kernels from each sample for mycological from each Division in each District were surface sterilized in 0.5% sodium hypochlorite solution for 30 seconds. The kernels were then rinsed in three changes of sterile distilled water to remove excess sodium hypochlorite. Then they were placed on sterile filter papers to for them to dry. Fungi were isolated from the maize by using Modified Rose Bengal Agar (MRBA) according to Cotty (1994). Five seeds were placed on the surface of Modified Rose Bengal Agar (MRBA) in five Petri-dishes replicated ten times and incubated at 25°C for five days. Isolation and determination of the incidence was done on MRBA on a completely randomized design (CRD). Isolates were identified as *A. flavus* based on colony characteristics, strain morphology and microscopic features according to Klich (2002). The incidences of *A. flavus* isolated from maize using MRBA in the four districts were enumerated according to Cotty (1994). Other species of *Aspergillus* isolated were also identified and their incindences determined. This was done by determining the percentage occurrence of each *Aspergillus* species from each Division in the 5 Districts.

3.4 Sub-culturing of *Aspergillus* species

A small mycelia plug of seven day old fungus on MRBA was transferred on to the surface of sterile PDA plates. The inoculated dishes were incubated at 25 - 27°C on the laboratory benches for seven days under alternating 12 hours of light and darkness. The spores from the pure cultures of *A. flavus* were harvested by adding double distilled sterile water and stored in universal bottles that had been sealed with parafilm. These were later used in the identification of the fungal isolates.

3.5 Identification of the fungal isolates

The fungal isolates were identified according to Klich (2002). This was done by sub-culturing the isolates onto Czapek Yeast Agar incubated at 25° C (CYA25). Fungal spore suspension made from 7 day old cultures on PDA was used. A 2µl aliqouts of the spore suspension was placed on three spots equidistant from the centre using sterile micro-pippetes on the surface of sterile CYA25. To prevent stray colonies inoculations were made from a spore suspension by pippeting 1ml aliquots into small glass vials that had been sterilized. The inoculated plates were incubated at 25°C in the incubator for seven days. Fungal isolates were identified according to Klich, (2002).

3.6 Collection of the medicinal plants

Fifteen medicinal plants used in this study were collected from Mwingi District. The plants included *Albizia antihelmintica, Balanites aegyptiaca* (L.) Del., *Boscia coriacea* Pax, *Carisa spiairum* L., *Croton megalocarpus, Maerua decumbens Forssk., Ricinus communis* L., *Psidium guajava, Salvadora persica* L., *Senna siamea, Tamarindus indica, Tithonia diversifolia, Zanthoxylem chalybeum, Solanum incanum* L., *and Melea volkensis.* These plants were identified in the herbarium of the Plant and Microbial Sciences Department, Kenyatta University, in which voucher specimens are deposited. Information about the plants is shown in the Table 3.1 and some photographs of the plants are shown in the plates 3.1a and 3.1b.

3.7 Extraction of the plant extracts

The leaves and the bark of the plants collected were shade dried and crushed into powder using a crushing machine (Christy, Type: 8 lab mill, Christy and Norris. England). Then

200g of the powder of each plant was mixed with 1 litre of methanol and placed in a shaker at 100 RPM for 24 hours. These were then passed through a sieve no.60 and filtered through a Whatman No. 1 filter paper and filter sterilized through a membrane filter (0.2µm) to avoid microbial contamination (Singh *et al.,* 1980). The filtrate was concentrated in a rotatory evaporator to produce a semi-solid residue that was further dried into powder form (Jonathan, 2002).

Table 3.1: Plants investigated for antifungal activities

Plant	**Kamba name**	**Family**	**Medicinal uses**
Albizia antihelmintica Brongn.	Kyowa	Fabaceae (Mimosaceae)	Bark used as vermifuge and vetirinary medicine
Balanites aegyptiaca (L.) Del.	Mulului (Kivuvwa)	Balanitaceae	Roots and fruits used as moluscicidal and kills fish
Boscia coriacea Pax.	Murui	Capparidaceae	Bark and roots used for vetirinary medicine and for disinfecting gourds
Carisa spinirum (Forssk.) Vahl.	Mukawa	Apocynaceae	Roots and branches used as veterinary medicine
Croton megalocarpus Hutch.	Muthulu	Euphorbiaceae	Bark used for veterinary medicine
Maerua decumbens (Brongni) De wolf.	Munatha	Capparidaceae	Roots and bark used to disinfect milk containers and fish poison

Melea volkensis Gürke.	Mukau	Meleaceae	Roots and branches used as veterinary medicine
Ricinus communis L.	Kivunu, (Mwaiki)	Euphorbiaceae	Roots and stem used for veterinary medcine
Psidium guajava L.	Kivela	Myrtaceae	Bark used as medicine for vermifuge and vetirinary medicine
Salvadora persica L.	Mukayau	Salvarodaceae	Roots and bark used for veterinary medicine
Senna siamea (*Cassia siamea*) Oliv.	Ikengeta	Fabaceae (Caesalpinaceae)	Roots and bark used as medicine, bark ground into powder to store grains
Solanum incunum L.	Mukondu	Solanaceae	Medicine for stomach pains, snake bites, tonsillitis, chest pains and skin wounds of cattle.
Tamarindus indica L.	Kithumula (Kikwasu)	Fabaceae (Caesalpinaceae)	Medicine for stomach pains, and vermifuge
Tithonia diversifolia (Hemsl.) A. Gray	Ilaa	Compositae (Asteraceae)	Medicine for stomach pains.
Zanthoxylem chalybeum Engl.	Mukenea (Mukanu)	Rutaceae	Leaves, bark and roots used as veterinary medicine, for diarrhoea in camels, and goat diseases, against coughs in camels and cattle

Plate 3.1a *Balanites aegyptiaca*

Plate 3.1b *Zanthoxylem chalybeum*

3.8 Phytochemical screening of the plant xetracts

The fifteen methanolic leaf extracts obtained were subjected to phytochemical screening methods according Oyetayo, (2008) and Sofowora, (1984) to determine the presence of bioactive agents such as tannins, alkaloids saponins, flavonoids, terpenoids and cardiac glycosides. For the bark extracts, phytochemical screening was done for the eight plants whose leaf extracts proved to be effective against *A. flavus*. The following methods were carried out to test for the presence of some of the bioactive compounds.

3.8.1 Test for tannins

A 200mg of each plant extract was dissolved in 10ml of distilled water and then filtered using Whatman No. 1filter paper. A 2ml of the filtrate then mixed with 2ml $FeCl_3$. Formation of blue-black precipitate indicated presence of tannins.

3.8.2 Test for saponin

The test was done using the frothing test. A 0.5 ml of the filtrate was mixed with 5ml distilled water.the formation of persistent frothing indicated the presence of saponins.

3.8.3 Test for flavonoids

Five millilitres of dilute ammonia solution were added to a portion of the aqueous filtrate of each plant extract followed by addition of 1ml of concentrated H_2SO_4. A yellow colouration observed in each extract indicated the presence of flavonoids. The yellow colouration disappeared on standing.

3.8.4 Test for terpenoids (Salkowski test)

In the Salkowski test, five ml of each plant extract was mixed with 2 ml of chloroform, and 3 ml concentrated H_2SO_4. was carefully added to form a layer. A reddish brown colouration of the inter-face was formed to show positive results for the presence of terpenoids.

3.8.5 Test for cardiac glycosides (Keller-Killani test)

In the Keller- Killani test, 2ml of each of the filtered plant extracts was mixed with 1ml of glacial acetic acid, $FeCl_3$ and 3ml of concentrated H_2SO_4. The formation of green-blue colour indicated the presence of cardiac glycosides.

3.8.6 Test for alkaloids

A 200mg of each plant extract was dissolved in 10ml of methanol and filtered. A 2ml of the filtrate then mixed with 3ml of Wagner’s reagent. The formation of an orange precipitate indicated the presence of alkaloids.

3.9 Determination of inhibition concentration

The antimicrobial activities of the plant extracts were determined using agar well diffusion technique (Ajaiyeoba *et al.*, 2003). The inoculums were prepared from actively growing *A. flavus* cultures that were 72 hours old by suspending them directly into sterile distilled water. These were later diluted to turbidity visually comparable to that of 1 McFarland standard (USP, 2006). Then Sabouraud Dextrose Agar (SDA) plates were seeded using a sterile glass L rod spreader with 25-µl mycelial suspensions of *A. flavus* that had been diluted to 1.0 McFarland Standards (CFU $x10^6$/mL). The seeded plates were allowed to set and then dry in the incubator at 37° C for 20 minutes. A standard aseptic cork borer of 7 mm diameter was used to cut 5 uniform wells on the surface of the agar. Then 60uL of each of the plant extracts at different concentrations of 400mg, 750mg and 1000mg of solid extracts dissolved in 1ml 85% methanol introduced into each well with the aid of 100 uL Pasture pippettes (Sama and Ajaiyeoba, 2006)with each treatment replicated five times. The plates seeded with the mycelial fungal suspension were incubated at 27° C for 72 hours after which diameters of zones of inhibition were measured. The plant extracts that produced an inhibition zone greater than 10mm were consindered to be effective (Sama and Ajaiyeoba, 2006). For the positive controls 10mg/ml of Meconazole was used according to the manufacturer's instructions while for the negative control, 85 % methanol was used. All the assays were carried out in five replicates. Diameters of the zones of inhibition of the plant extracts were determined as an indication of antifungal activity.

3.10 Determination of the minimum inhibition concentration (MIC) and minimal fungicidal concentration (MFC)

The MIC and the MFC were applied to the plant extracts that proved to be effective against *A. flavus* in the agar well diffusion method at 400mg/ml determined according to Rasooli and Abyaneh, (2004). The MIC was aimed at finding the lowest concentration of the extract that will inhibit the growth of *A. flavus.* It was done using SDB broth micro-dilution method where different concentrations of 1.56mg/ml, 3.125 mg/ml, 6.25 mg/ml and 25mg/ml of the extracts each replicated three times were prepared. A 5ml of sterile extracts at different concentrations were taken into sterile empty 10ml Durham tubes. Then 1ml of a 72 hours old culture of *A. flavus* was added into the extracts and mixed. Then 1ml of this mixture was added into 5ml of sterile Sabouraud Dextrose Broth (SDB) in tubes. All the tubes were incubated at 30°C for 15 days and observations made for presence of visible growth of fungi. The highest dilution without visible growth was regarded as the Minimum Inhibitory Concentration (MIC) during 15 days. The cell cultures from the tubes without growth were then sub-cultured on Sabouraud Dextrose Agar (SDA) plates and incubated at 30^{0}C for 5 days. This was to determine if the inhibition was reversible and to determine the Minimal Fungicidal Concentration (MFC).

The MFC was determined as the highest dilution at which no growth occurred on the SDA plates. For the positive controls, 1ml of the mixture (Meconazole +*A.flavus* culture) added into SDB without the plant extracts. For the negative control 1ml the mixture was added into 5ml SDB without the plant extract. All the treatments were done in triplicates. The tubes with the highest dilution (lowest concentration) that showed no visible growth in the SDB were recorded as MIC. The cultures from the highest dilution (lowest concentration)

that produced no growth on the SDA plates were recorded as the MFC (Irkin and Korukluoglu, 2007).

3.11. Aflatoxin analysis

3.11.1 Sample preparation

Sample preparation and aflatoxin analysis were done in the Botany Department laboratory at University of Nairobi. The whole sample of maize for mycological analysis from each Division was ground with a Romer mill (Union, IL, USA). A 50g sub- sample of the milled maize flour was mixed with 250 ml of methanol/water (60: 40, v/v) and the mixture vortexed at high speed for 3 minutes. This was followed by centrifugation at 3600 rpm for 10 minutes and the recovery of 125ml of the supernatant filtered (Whatman filter paper, No.1) into a separatory funnel. The mixture was then defatted by adding 30 ml saturated sodium chloride solution and 50 ml of hexane on a vortex for 30 seconds and left to separate. The methanol content of the extract was diluted to 10% using phosphate buffer solution (PBS) before analysis by Enzyme Linked Immunosobent Assay (ELISA).

3.11.2 ELISA

Directly competitive ELISA method using micro-titer plates as described by Hongyo *et al.,* (1992) for aflatoxin analysis was used. The method involved coating micro-titer plates overnight at room temperature with 100µl of anti-aflatoxin antiserum for aflatoxin analysis diluted 1: 15,000 IN 0.1m Sodium Carbonate buffer. Free protein binding sites of the wells were blocked with 200µl of 3% fetal calf serum in PBS for 30 minutes at room temperature. Then the plates were washed in saline solution containing 0.05% tween 20,

aliquots of 50µl of standard aflatoxin B solution and sample extract solutions in MeOH/PBS (10%) were incubated simultaneously with 50µl of AFB1-HRT in 1% fetal calf serum in PBS for two hours at room temperature. The plates were washed and 100 µl substrate solution containing tetra-methyl benzidine and H_2O_2 added to each well. After 7 - 10 minutes, the enzyme reaction was stopped with 100l of 1M H_2SO_4 in each of micro-titer well. The absorbance of the resultant colour was measured at 450nm using Uniskan II micro-plate reader (Labsystems, Finland). To quantify the toxin content in the samples, a standard curve was made using the absorbent values of standards (Y-axis) and corresponding standard concentration (X-axis) used by using curve fitting programmed (In-plot Graphics) using absorbance value samples. The toxin content in the sample extracts was deduced by interpolation from the standard curve. The value obtained for sample extracts were multiplied with the dilution factor to obtain the toxin content in the samples.

3.12 Statistical analysis

Statistical analyses involved transformation of the percentage data on the incidences of *A flavus* the using the square root method. The means for incidences of *Aspergillus* species and the zones of inhibition of the plant extracts were separated using ANOVA and the data analysed by Tukey's test at $p< 0.05$.

CHAPTER 4

RESULTS

4.1 Isolation and identification of fungal isolates from maize samples collected from Eastern Kenya

The maize used in the study was collected from 5 Districts in Eastern Kenya. It was sampled from different maize storage structures that are commonly used by the local farmers. Some of these structures are shown in Plates 4.1a – 4.1f.

Plate 4.1a: An all round grass granary for maize sotrage

Plate 4.1b: Wooden grass roofed granary for maize storage

Plate 4.1c: Wooden grass roofed granary for maize sotrage

Plate 4.1d: Sisal stalk iron roofed granary for maize sotrage

Plate 4.1e: Wooden iron roofed granary the for maize sotrage

Plate 4. 1f: Maize storage by heaping on floor

Four different different *Aspergillus* species; *Aspergillus flavus*, *Aspergillus niger*, *Aspergillus ochraceous* and *Aspergillu*s *tamarii* were isolated from the maize samples from the 5 Districts.

The isolates of *A. flavus* formed yellow green colonies on MRBA (Plate 4.2a). On CYA25, the morphological features revealed that, *A. flavus* forms mycelia that are white with spreading yellow green colonies; crowns of conidia that are olive green in colour and that are overlaid with olive yellow areas. The fungus produces dark brown sclerotia and uncoloured exudates. A colony diameter of 64mm is attained by the fungus at day 7. The microscopic characteristics of the fungi included long and septate cells borne on hyaline hyphae, colourless conidiophores that are wide and roughened, globose vesicles held on long conidiophores, and smooth globose conidia. The subculture of *A. flavus* on PDA is shown on Plate 4.2b.

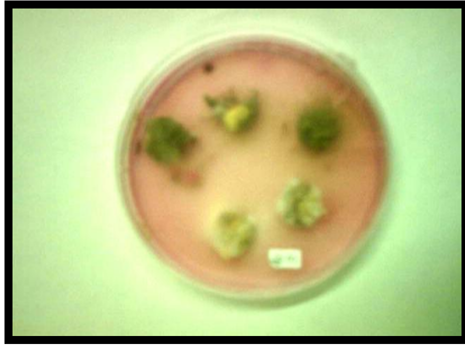


Plate 4.2a: *A. flavus* isolated on MRBA.

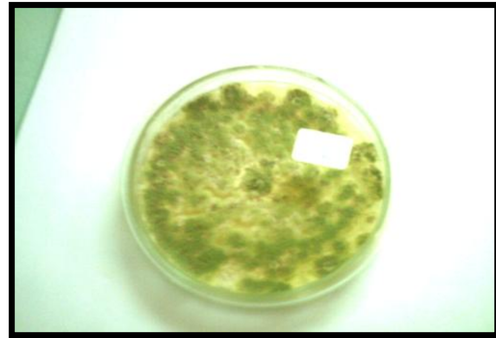


Plate 4.2 b: Sub-Culture of *A flavus* on PDA

The isolates of the fungus *A. ochraceous* formed pale yellow colonies on MRBA (Plate 4.2c). The fungi formed dense conidia on short inconspicuous white mycelia. The morphological characteristics of the fungi revealed on CYA25 included yellowish conidia that are densely formed, low (short) white and inconspicuous mycelia that do not spread, uniform colony texture, colourless exudates and dull pink sclerotia, and colony diameter of 56mm at day 7. The microscopic characteristics included smaller and finely roughened conidia, globose and biseriate vesicles, long and septate hyphae and narrow stipes. The subculture of *A. ochraceous* on PDA is shown on Plate 4.2d.

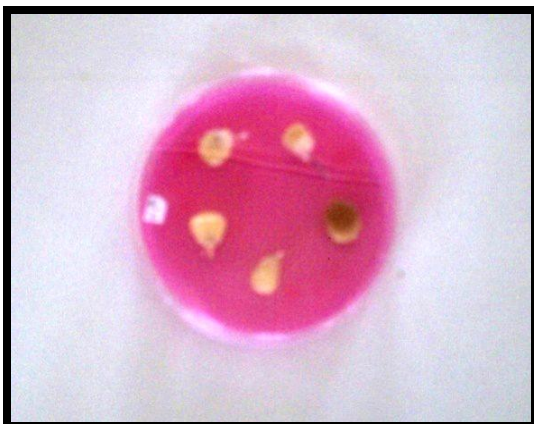


Plate 4.2c: *A. ochraceous* isolated on MRBA

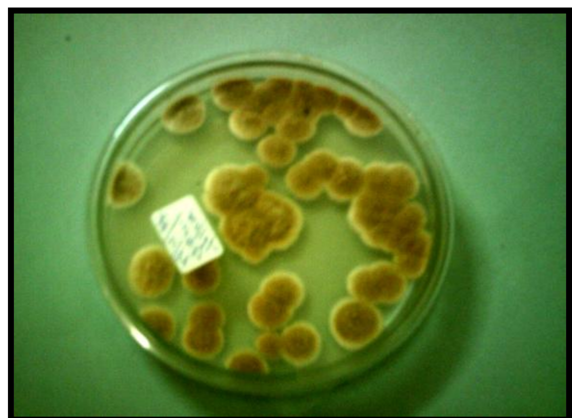


Plate 4.2d: Sub-Culture of *A. ochraceous* on PDA

The isolates of the fungus *A. niger* formed black colonies on MRBA (Plate 4.2e). The conidia are black and produced on colourless hyphae. The morphological characteristics revealed the fungus to have colony diameter of 62mm at day 7, black conidia that are densely packed, white inconspicuous hyphae, rough colony texture, uncoloured exudates and a light yellow reverse. The microscopic characteristics included thick walled stipes, radiate and biseriate conidia. The subculture of *A. niger* on PDA is shown on 4.2f.

Plate 4.2e: *A. niger* isolated on MRBA.

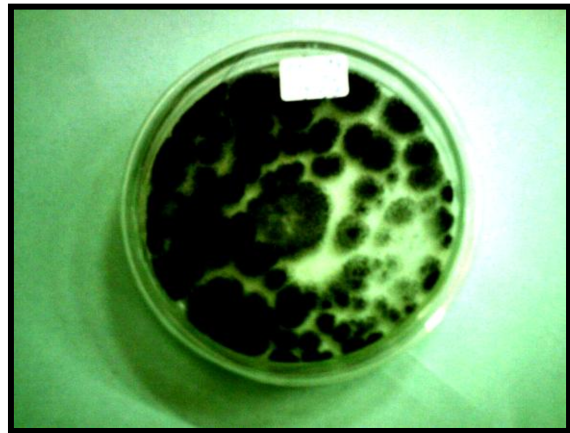


Plate 4.2f: Sub-Culture of *A. niger* on PDA

The isolates of the fungus *A. tamarii* formed small colonies of deep brown colour on MRBA (Plate 4.2g). They produced brown conidia heads on white inconspicuous mycelia. The colonies have rough texture due to the uneven stipes. The morphological features of the fungi on CYA25 revealed olive brown conidia, white and inconspicuous mycelia, very large uncolored conidia heads, and uneven stipes that gives the colony a coarse texture, light yellow exudates and grayish yellow reverse. Microscopic characteristics of the fungi included radiate and biseriate conidia heads; rough walled and uncoloured stipes and large, rough and thick walled conidia. The subculture of *A. tamarii* on PDA is shown on Plate 4.2h.

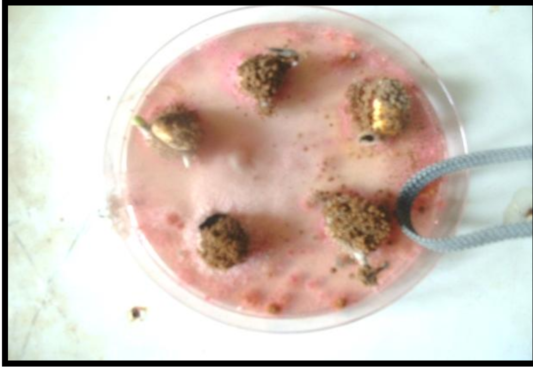


Plate 4.2g: ***A. tamarii*** **isolated on MRBA.**

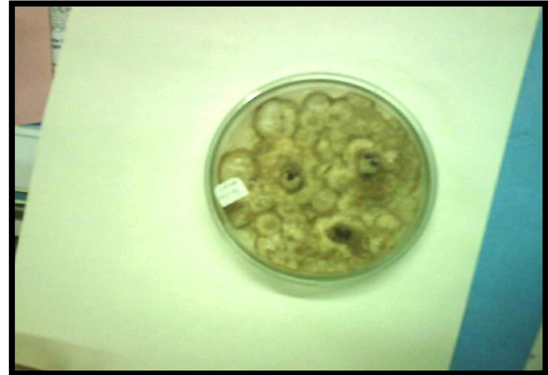


Plate 4.2h: Sub-culture of ***A. tamarii*** **on PDA**

4.2 Incidences of *Aspergillus* sp. in stored maize in Eastern Province

All the four *Aspergillus* sp. that include *A. flavus, A. niger, A. ochraceous* and *A. tamarii* were found contaminating the stored maize sampled from all the 10 Divisions (Central, Kathiani, Kangundo, Matungulu, Kibwezi, Makindu, Mutomo, Mutha, Kaiti and Wote) in 5 Districts from Eastern Province, (Kangundo, Kibwezi, Kitui South, Makueni and Machakos Districts) were determined in the department of Plant and Microbial Sciences' laboratory at Kenyatta University.

4.2.1 Incidences of total *Aspergillus* sp. from the 5 Districts in Eastern Province

The incidences of the total *Aspergillus* sp. in the 5 Districts were detected to vary significantly ($F = 6.13$, $df = 4$, $P < 0.05$). Machakos and Kangundo had high incidences of 58.80% and 58.4 % respectively while Kibwezi had lowest of 24.20 % (Figure 4.1).

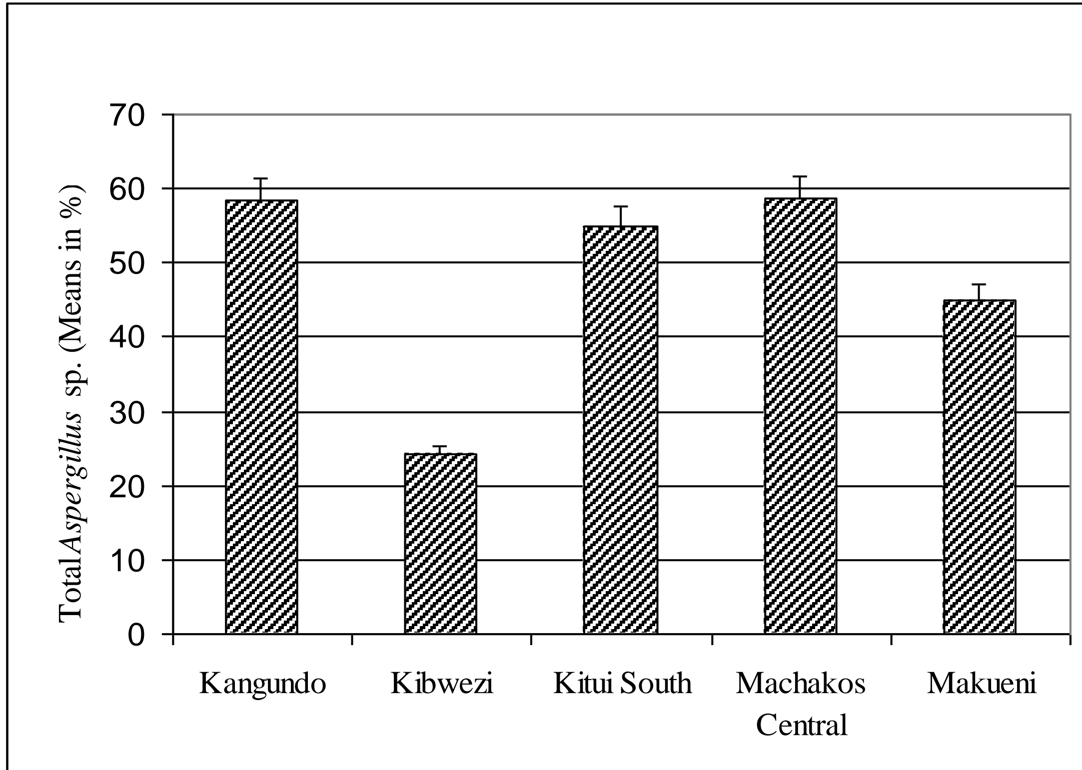


Figure 4.1: Incidences of the total *Aspergillus* sp. in maize from the 5 Districts

4.2.2 Incidences of total *Aspergillus* sp. from the 10 Divisions in Eastern Province

sp. isolated from the 10 Divisions were detected to vary significantly ($F = 2.76$, $df = 9$, $p < 0.05$) in the Divisions. Central (mean = 58.80 %) and Kathiani (mean = 58.80 %) Divisions had significantly higher incidences than the others. Kibwezi (mean = 19.20 %) was noted to have significantly low incidences of *Aspergillus* sp than in all the other divisions (Table 4.1).

Table 4.1: Incidences of the total *Aspergillus* sp. from the 10 Divisions in Eastern Province

Divisions	Total *Aspergillus* sp. (Means in %)
Central	58.80a
Kaiti	51.60a
Kangundo	58.40a
Kathiani	58.80a
Kibwezi	19.20b
Makindu	29.20ab
Matungulu	58.40a
Mutha	56.80a
Mutomo	52.80a
Wote	38.40ab

Note: Numbers are means of 10 replications. Means are separated by Turkeys Test. Numbers followed by the same letters are not significantly different ($P < 0.05$).

4.2.3 Incidences of *Aspergillus* sp. in stored maize from the five Districts

The incidences of *Aspergillus* species from stored maize sampled from Kangundo District, indicated a significant difference ($F = 24.63$, $df = 3$, $P < 0.05$) for the respective species. The incidence of *A. flavus* (mean = 31.71 %) in the District was significantly higher than of the other three fungi in which, *A. niger (*mean = 14.32 %), *A. ochraceous* (mean = 6.93 %) and *A. tamarii* (mean = 2.72 %) (Table 4.2).

The study, however detected that there was no significant difference in the incidences of the fungi in the two Divisions (Matungulu and Kangundo Divisions) in Kangundo District. Although the overall incidence of the fungi showed that there were more *Aspergillus* sp. in

maize samples from Kangundo (mean = 12.17 %) than from Matungulu (mean = 11.30 %) Division.

In Kibwezi District, the incidences of *Aspergillus* species isolated from stored maize significantly differed ($F = 26.06$, $df = 3$, $P < 0.05$). The fungus *A. flavus* (mean = 15.21 %) recorded significantly high incidence than the other three fungi that had significantly low incidences; *A. niger* (mean = 3.98 %), *A. ochraceous* (mean = 2.69 %) and *A. tamarii* (mean = 0.723 %) (Table 4.2).

Comparing the incidences of *Aspergillus* species in the two Divisions in Kibwezi District, Makindu (mean = 27.56 %) had more of the *Aspergillus* sp. than Kibwezi (mean = 12.39 %).

The incidences of *Aspergillus* species in maize samples collected from Makueni District indicated a significant difference ($F = 27.29$, $df = 3$, $P < 0.05$) for the respective species. *A. flavus* (mean = 25.53 %) was more than *A. ochraceous* (mean = 7.13 %), *A. niger* (mean = 4.85%) and *A. tamarii* (mean = 4.71 %) (Table 4.2).

Comparing presence of the *Aspergillus* species in the two Divisions in Makueni District, there was no significant difference even though Kaiti Division (mean = 9.99 %) had a higher incidence than Wote Division (mean = 8.33 %).

The incidences of *Aspergillus* species isolated from stored maize samples collected from Kitui South District showed that there was a significant difference ($F = 21.78$, $df = 3$, $P < 0.05$) for rhe different *Aspergillus* species. In this District, *A. tamarii* (mean = 27.4 %) had

a higher incidence than *A. flavus* (mean = 21.37 %), *A. niger* (mean = 2.63 %) and *A. ochraceous* (mean = 1.197 %) (Table 4.2).

A comparison of the presence of the fungi in the two Divisions in Kitui South District indicated no significant difference even though Mutha Division (mean = 11.59 %) had a higher incidence than Mutomo Division (mean = 7.33 %).

There was a significant difference ($F = 12.14$, $df = 3$, $P < 0.05$) in the incidences of the different *Aspergillus* species isolated from stored Maize samples collected from Machakos District. The results in this District, showed that, *A.tamarii* (mean = 22.20 %) had a high incidence than *A. flavus* (mean = 12.92 %), *A. ochraceous* (mean = 4.24 %) and *A. niger* (mean = 4.80 %) (Table 4.2).

Comparing presence of the fungi in the two Divisions in Machakos District, there was no significant difference even though Kathiani Division (mean = 11.35 %) had a higher incidence than Central Division (mean = 8.29 %).

Table 4.2: Incidences of fungi in the *Aspergillus* sp. from the 5 Districts in Eastern Province

***Aspergillus* sp.**	**Incidence of *Aspergillus* sp. (Means in %)**				
	Kangundo	**Kibwezi**	**Makueni**	**Kitui**	**Machakos**
A.niger	14.32b	3.98ab	4.85a	2.63a	4.80a
A.tamarii	2.72ab	0.723ab	4.71a	27.40ab	22.20ab
A. flavus	31.71c	15.21b	25.53b	21.37ab	12.92ab
A. ochraceous	6.93ab	2.69ab	7.13a	1.197a	4.24a

Note: Numbers are means of 10 replications. Means are separated by Turkeys Test. Numbers followed by the same letters in the same column are not significantly different ($P < 0.05$).

4.2.4 Incidences of *Aspergillus* sp. from stored maize in the 10 Divisions

In Kangundo District, maize was sampled from two Divisions that included Matungulu and Kangundo. The results of the incidences of *Aspergillus* species from Matungulu Division indicated a significant difference ($F = 21.41$, $df = 3$, $P > .05$). The incidence of *A. flavus* (mean = 32.06 %) was significantly higher than the other fungi (Table 4.3).

In Kangundo Division, the incidences of *Aspergillus* species showed that there was a significant difference ($F = 11.10$, $df = 3$, $P > .05$) in the incidences of the different fungi. The incidence of *A. flavus* (mean = 30.25 %) was significantly higher than of the other three species (Table 4.3).

In Kibwezi District, maize was sampled from two Divisions that included Kibwezi and Makindu. The incidences of *Aspergillus* species in Kibwezi Division varied significantly for the different fungi isolated (F =24.47, df =3, P<0.05). The incidence of *A. flavus* (mean = 13.48 %) was significantly higher than in all the other species. The incidence of *A. tamarii* (mean = 0.72 %) was significantly low (Table 4.3).

The incidences of the different fungi in the *Aspergillus* species isolated from maize from Makindu Division significantly differed (F = 9.43, df =3, P<0.05). The incidence of *A. flavus* (mean = 17.06 %) was significantly higher than in all the other species. The incidence of *A. tamarii* (mean = 0.72 %) was significantly low (Table 4.3).

In Makueni District, maize was sampled from two Divisions that included Wote and Kaiti. The incidences of *Aspergillus* species in Wote Division varied significantly in the different species (F = 16.70, df =3, P<0.05). The incidence of *A. flavus* (mean = 19.61 %) was significantly higher than in all the other species. The incidence of *A. ochraceous* (mean = 5.57 %) was significantly low (Table 4.3).

The incidences of *Aspergillus* species in Kaiti Division were found to vary significantly (F = 27.39, df =3, P<0.05). The incidence of *A. flavus* (mean = 32.8 %) was significantly higher than in all the other species with *A.tamarii* recording the lowest incidence (mean = 2.55 %) (Table 4.3).

In Kitui South District, maize was sampled from Mutomo and Mutha Divisions. In Mutomo Division, the incidences of the different *Aspergillus* species isolated showed that

there was a significant difference (F = 8.99, df =3, P<0.05). The incidence of *A. tamarii* (mean = 33.2 %) was significantly higher than in all the other species. The incidence of *A .ochraceous* (mean = 00 %) was significantly low (Table 4.3).

The incidences of fungi in the *Aspergillus* species in Mutha Division differed significantly (F = 26.46, df =3, P<0.05). The incidence of *A. flavus* (mean = 25.72 %) was significantly higher than in all the other species. The incidence of *A .ochraceous* (mean = 2.18 %) was significantly low (Table 4.3).

In Machakos District, maize was sampled from two Divisions that included Machakos Central and Kathiani. In Machakos Central Division, the incidences of *A. flavus* (mean = 15.21 %) and *A.tamarii* (mean = 13.87 %) were significantly higher than in the other two species (F = 12.03, df =3, P<0.05**)**. The incidence of *A. ochraceous* (mean = 1.598 %) was significantly low (Table 4.3).

The incidences of *Aspergillus* sp. in Kathiani Division differed significantly (F = 8.12, df =3, P<0.05). The incidence of *A. tamarii* (mean = 35.14 %) was significantly higher than in all the other species. The incidence of *A .niger* (mean = 3.86 %) was significantly low (Table 4.3).

Table 4.3: Incidences of different species of *Aspergillus* in stored maize in the 10 Divisions

Divisions	Incidence of *Aspergillus* sp. (Means in %)			
	A. flavus	***A .niger***	***A .ochraceous***	***A .tamarii***
Matungulu	32.06a	15.13b	5.68bc	1.98c
Kangundo	30.25a	13.53b	8.29bc	3.39c
Kibwezi	13.48a	3.03b	1.61b	0.72b
Maikindu	17.06a	4.71b	4.05b	0.72b
Wote	19.61a	4.96b	5.57b	7.54b
Kaiti	32.8a	4.74b	9.94b	2.55b
Mutomo	17.44a	0.72b	00b	33.20a
Mutha	25.72a	5.74b	2.18b	21.81a
Machakos	15.21a	5.26b	2.55b	13.87a
Kathiani	15.37a	3.86a	6.36a	35.14b

Note: Numbers are means of 10 replications. Means are separated by Turkeys Test. Numbers followed by the same letters in a row are not significantly different ($P < 0.05$).

4.3 The incidences of *A. flavus* from stored maize in Eastern Province

4.3.1 The incidences of *A. flavus* from stored maize in the 5 Districts

There was no significant difference in the incidences of *A. flavus* isolated from stored maize collected from all the 5 Districts in Eastern Province ($F = 7.73$, $df = 4$, $P < 0.05$). Kangundo District (mean = 31.71 %) had higher incidence of *A. flavus* than Makueni (mean = 25.53 %), Kitui South (mean = 21.37 %), Kibwezi (mean = 15.22 %) and Machakos District (mean = 12.92 %) (Table 4.4).

Table 4.4: Incidences of *A. flavus* in maize from the 5 Districts in Eastern Province

District	**Incidence of *A. flavus* (Means in %)**
Kangundo	31.71a
Kibwezi	15.22b
Kitui South	21.37ab
Machakos	12.92ab
Makueni	25.53ab

Note: Numbers are means of 10 replications. Means are separated by Turkeys Test. Numbers followed by the same letters are not significantly different ($P < 0.05$).

4.3.2 Incidences of *A. flavus* from stored maize from the 10 Divisions in the 5 Districts

Analysis of the incidences of *A. flavus* in all 10 Divisions in the 5 Districts revealed a significant difference ($F = 4.91$, $df = 9$, $P < 0.05$). This particular species of *Aspergillus* had a higher incidence (mean = 33.18 %) in Matungulu Division. The lowest incidence was detected in Kathiani Division (mean = 10.82 %) (Table 4.3).

4.4 The incidences of *A. niger* from stored maize in Eastern Province

4.4.1 Incidences of *A. niger* from stored maize in the five Districts in Eastern Province

There was a significant difference ($F = 6.87$, $df = 4$, $P < 0.05$) in the incidences of *A. niger* in the 5 Districts. The incidence of *A. niger* was higher in Kangundo (mean = 14.32 %) than in the other Districts which recorded significantly low incidences of the fungi (Table 4.5).

Table 4.5: Incidences of *A. niger* in maize from the 5 Districts in Eastern Province

District	Incidence of *A. niger* (Means in %)
Kangundo	14.32a
Kibwezi	3.98b
Kitui South	2.63b
Machakos	4.53b
Makueni	4.85b

Note: Numbers are means of 10 replications. Means are separated by Turkeys Test. Numbers followed by the same letters are not significantly different ($P < 0.05$).

4.4.2 Incidences of *A. niger* in maize from the 10 Divisions in the 5 Districts

There was a significant difference ($F = 3.97$, $df = 9$, $P < 0.050$) in the incidences of *A. niger* in the 10 Divisions. *A. niger* had a higher incidence in Matungulu (mean = 15.13 %) and Kangundo (mean = 13.54 %) than in the other Divisions (Table 4.3).

4.5 The incidences of *A. ochraceous* from stored maize in Eastern Province

4.5.1 Incidences of *A. ochraceous* in maize from the 5 Districts

There was a significant difference ($F = 7.44$, $df = 4$, $P < 0.05$) in the incidences of *A. ochraceous* in maize in the 5 Districts sampled. The incidence of *A. ochraceous* was higher in Makueni (mean = 7.11 %) than in the other Districts (Table 4.6).

Table 4.6: Incidence of *A. ochraceous* in maize from the 5 Districts

District	Incidence of *A. ochraceous*. (Means in %)
Kangundo	6.93a
Kibwezi	2.69b
Kitui South	1.197c
Machakos	4.24ab
Makueni	7.13a

Note: Numbers are means of 10 replications. Means are separated by Turkeys Test. Numbers followed by the same letters are not significantly different ($P < 0.05$).

4.5.2 Incidences of *A. ochraceous* in maize from the 10 Divisions Eastern Province

There was a significant difference ($F = 6.00$, $df = 9$, $P < 0.05$) in the incidences of *A. ochraceous* in the 10 Divisions sampled. Kaiti (mean = 9.94 %) and Kangundo (mean = 8.29 %) had higher incidence of *A. ochraceous* than the other Divisions with the lowest recorded in Mutomo (mean = 0.50 %) (Table 4.3).

4.6 The incidences of *A. tamarii* from stored maize in Eastern Province

4.6.1 Incidences of *A. tamarii* in maize from the 5 Districts

There was a significant difference in the incidences of *A. tamarii* in the 5 Districts ($F = 7.97$, $df = 4$, $P < 0.05$). *A. tamarii* had a higher incidence in Kitui South (mean = 27.4 %) than in the other Districts (Table 4.7).

4.6.2 Incidence of *A. tamarii* in maize from the 10 Divisions in Eastern Province

There was a significant difference in the incidences of *A. tamarii* in the maize sampled from the 10 Divisions ($F = 10.15$, $df = 9$, $P < 0.05$). Kathiani (mean = 35.14 %) and Mutomo (mean = 25.91 %) had significantly high incidences of *A. tamari* than the other Divisions (Table 4.3).

Table 4.7: Incidences of *A. tamarii* in maize from the 5 Districts

District	**Incidence of *A. tamarii* (Means in %)**
Kangundo	2.72a
Kibwezi	0.72b
Kitui South	27.4c
Machakos	22.74c
Makueni	4.71a

Note: Numbers are means of 10 replications. Means are separated by Turkeys Test. Numbers followed by the same letters are not significantly different ($P < 0.05$).

4.7 Determination of the Aflatoxin levels in stored maize from Eastern Provinnce

The aflatoxin levels (ppb) in maize samples from the 10 Divisions in the 5 Districts were found to vary. Kibwezi (60.35ppb) and Kathiani (50ppb) had the highest aflatoxin levels. Kangundo (0ppb), Mutha (5.5ppb), Wote (7.01ppb) and Kaiti (10.5ppb) had the lowest afltoxin levels (Figure 4.6).

Correlation was determined to find out the relationship between the incidence of *A. flavus* and aflatoxin levels in stored maize in the 10 Divisions. There was a highly significant correlation between the incidences of *A. flavus* and aflatoxin levels in the maize samples

analyzed (r = -0.758, P = 0.01). Out of the10 samples analyzed, 5 had > 20 ppb of aflatoxin levels. Only 2 samples had > 50 ppb of aflatoxin levels (Figure 4.7).

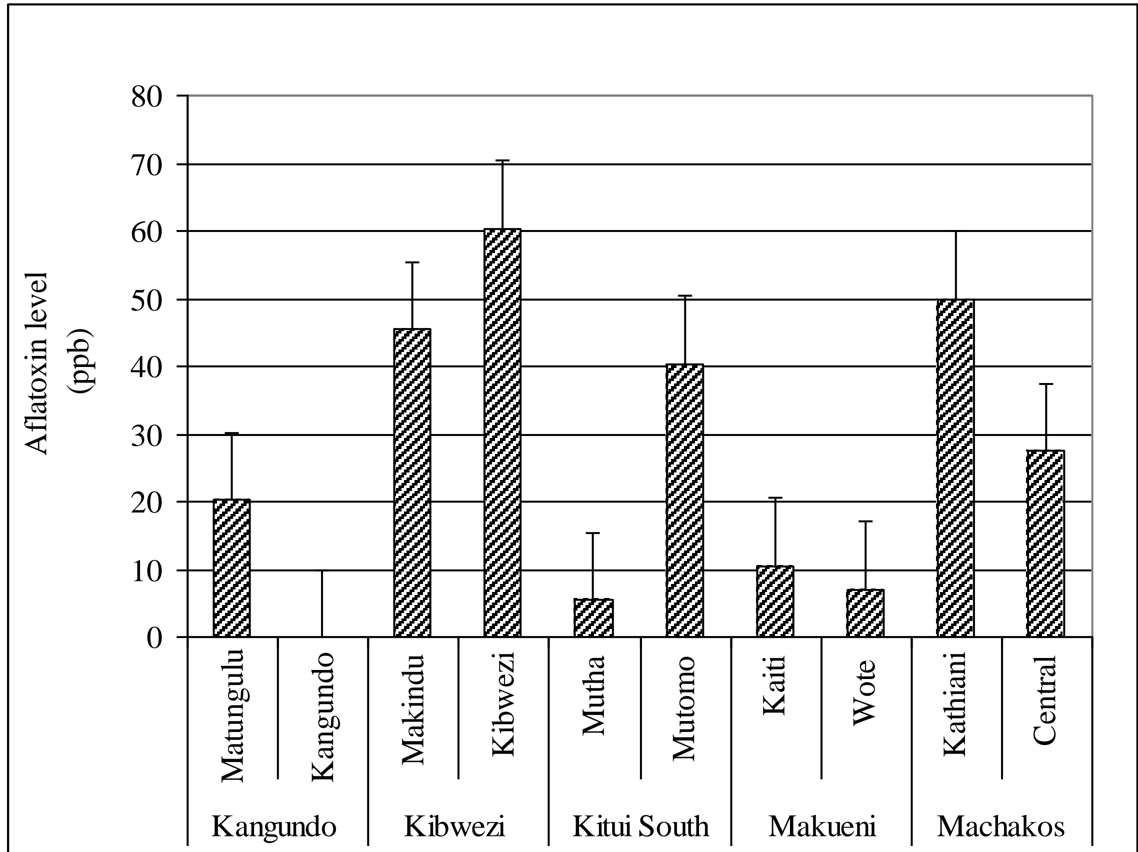


Figure 4.6: Aflatoxin levels in maize sampled the 10 Divisions in the 5 Distrcts

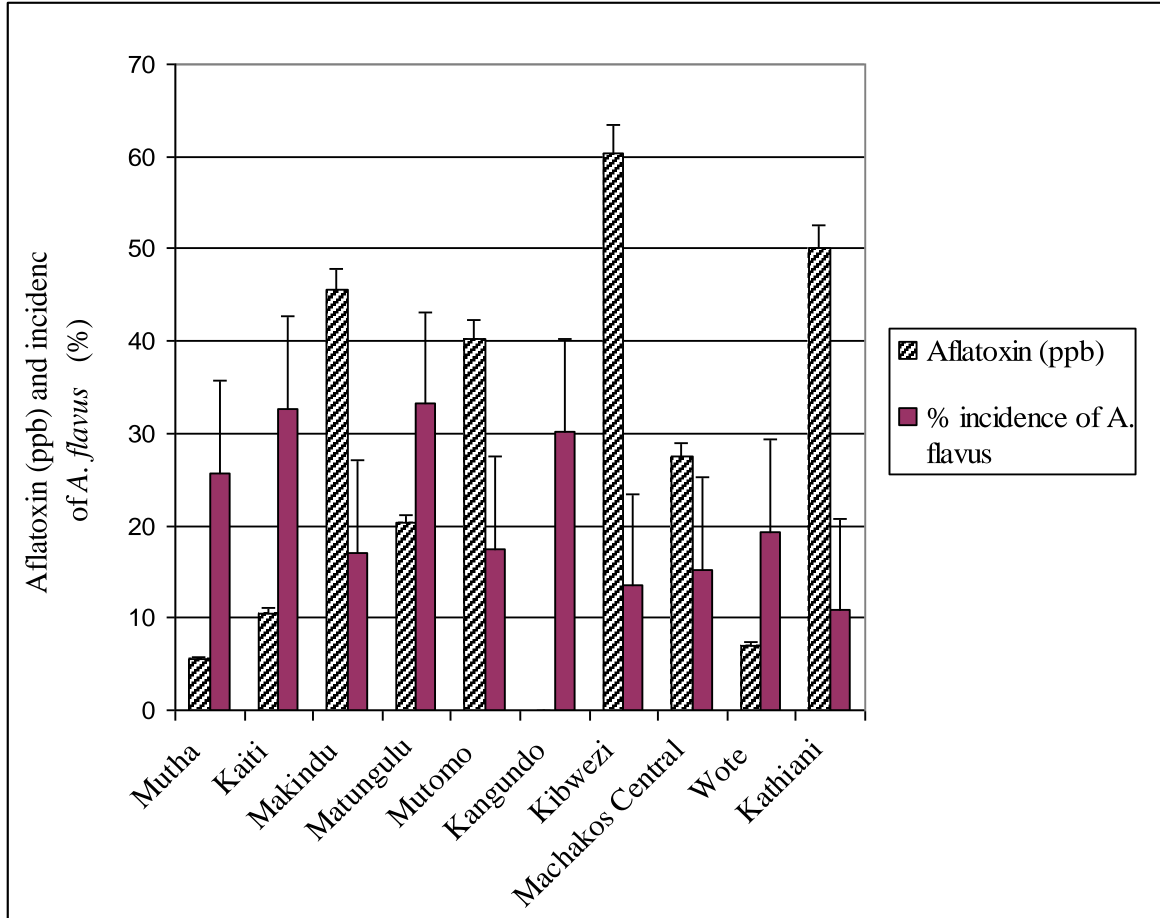


Figure 4.7: Correlation between aflatoxin levels and incidence of *A. flavus* in the 10 Divisions

4.8 Phytochemical screening of the medicinal plants

The results of the preliminary phytochemical screening of the fifteen methanolic plant leaf extracts revealed the presence of various bioactive agents. The bark extracts of the plants found to have effective leaf extracts were also analysed for the presence of bioactive agents. Both the leaf and bark extracts were found to contain agents such as tannins, flavonoids, terpenoids, cardiac glycosides, saponins and alkaloids (Tables 4.8 (a) and 4.8 (b)). The key below the tables gives the codes and the names of the medicinal plants.

Table 4.8(a): Bioactive agents screened from leaves of the medicinal plants

Plant code	Tanins	Saponins	Flavonoids	Terpenoids	Cardiac glycosides	Alkaloids
01	+ +	–	++	+ +	+ +	+ +
02	–	–	+ +	–	–	+
03	–	–	+ +	+ + +	+ +	+ +
04	+++	+++	–	–	–	++
05	+++	–	+++	+++	–	–
06	–	–	++	–	+	–
07	++	–	+	–	–	+++
08	+++	–	++	+++	–	–
09	+	–	+++	+++	–	–
10	–	–	–	–	–	+++
11	–	–	+	–	–	+
12	+	–	–	–	–	+++
13	++	–	+++	+++	–	+++
14	++	–	++	++	+	+++
15	–	–	–	+	+	+++

Table 4.8(b): Bioactive agents of extracts from the bark of the medicinal plants

Plant code	Tanins	Saponins	Flavonoids	Terpenoids	Cardiac glycosides	Alkaloids
03	–	–	+++	–	++	+++
04	+++	+++	–	–	+	+++
05	–	–	–	+++	–	+++
07	+++	–	+++	–	–	–
08	–	++	–	–	+	+++
09	–	—	+++	–	++	+++
11	–	–	++	–	–	+++
16	+++	+++	+++	–	–	+++
12	++	–	–	–	–	+++
13	++	–	+++	++	–	+++
14	++	–	++	++	–	+++
15	+++	–	+++	–	–	+++

Key:

Code	Plant	Code	Plant		
01	*A. antihelmintica*	**09**	*S. siamea*	+++	= Abundant
02	*B. aegyptiaca*	**10**	*S. persica*	+ +	= Moderate
03	*B. coriacea*	**11**	*T. indica*	+	= Trace
04	*C. spirinum*	**12**	*T. diversifolia*	-	= Negative
05	*C. megalocarpus*	**14**	*S. incunum*		
06	*M. decumbens*	**13**	*Z. chalybeum*		
07	*R. communis*	**15**	*M. volkensis*		
08	*P. guajava*				

4.9 Determination of the efficacy of plant extracts on the growth of *A. flavus*

Fifteen plant samples were tested for their effects on the growth of *A. flavus* in vitro in the laboratory of PMS department at Kenyatta University. The crude extracts of these plants exhibited good antifungal activity against *A. flavus* with varied zones of inhibition at different concentrations. An inhibition zone of a diameter of 10 mm and above was taken to be effective (Sama and Ajaiyeoba, 2006). Some of the plant extracts had large inhibition zones at different concentrations that compared favourably with those obtained with the standard antifungal agent meconazole (10 mg/ml).

4.9.1 Efficacy of plant leaf extracts on the growth of *A. flavus* at the different concentrations

The plant leaf extracts at 1000 mg/ml inhibited the growth of the fungi differently. The results showed that plant extracts of *B. coricea* (mean = 24.40 mm), *C. megalocarpus* (mean = 23.20 mm), and *S. incunum* (mean = 21.00 mm), *T. diversifolia* (mean = 20.00 mm) had significantly ($F = 27.59$, $df = 15$, $P < 0.05$) high inhibitory effect on the growth of *A. flavus* (Table 4.9).

Inhibition of the growth of *A. flavus* by the plant extracts at 750 mg/ml differed significantly ($F = 94.08$, $df = 15$, $P < 0.05$) with variations in the type of plants used. The study established that, the most effective plant extracts were from *B. coriacea* (mean = 19.40 mm), *C. megalocarpus* (mean = 19.00 mm) and *S. siamea* (mean = 17.20 mm). Plant leaf extracts from *M. decumben* and *S. persica* were not effective at 750 mg/ml concentration (Table 4.9).

Inhibition of the growth of *A. flavus* by the plant leaf extracts at 400 mg/ml significantly differed (F = 100.70, df = 15, $P < 0.05$) with variations in the type of plants used. The study established that the most inhibitory plant extracts were from *B. coriacea* (mean = 17.40 mm), *Z. chalybeum* (mean = 17.20 mm). Plant extracts from *A. antihelmintica, M. decumben* and *S. persica* did not inhibit growth of *A. flavus* at this concentration (Table 4.9).

Table 4.9: Inhibition of growth of *A. flavus* by plant leaf extracts at the different concentrations

Plants	Inhibition zones (mm) 1000 mg/ml	Inhibition zones (mm) at 750 mg/ml	Inhibition zones (mm) at 400 mg/ml
A. antihelmintica	15.20bc	12.60b	0.00a
B. aegyptiaca	12.20c	9.80c	7.00b
B. coriacea	24.40a	19.40a	17.40c
C. spirinum	13.80bc	15.20b	16.40c
M. decumben	7.00d	0.00d	0.00a
R. communis	12.20c	10.60c	7.00b
P. guajava	14.60bc	15.20b	16.20cd
S. siamea	19.80b	17.20a	15.20d
S. persica	7.00d	0.00d	0.00a
T. indica	12.20b	10.80c	7.00b
T. diversifolia	20.00a	17.00a	15.40cd
Z. chalybeum	19.00b	18.20a	17.20d
S. incunum	21.00a	16.60ab	15.80a
M. volkensis	17.80b	14.20b	7.00b
C. megalocarpus	23.20a	19.00a	15.60cd
Positive control (10mg/mm)	19.40b	18.40a	18.20c

Note: Numbers are means of 10 replications. Means are separated by Turkeys Test. Numbers followed by the same letters in the same column are not significantly different ($P < 0.05$).

The comparison of the efficacy of the leaf extracts of the fifteen plants at the three different concentrations is shown in Figures 4.8a and 4.8b.

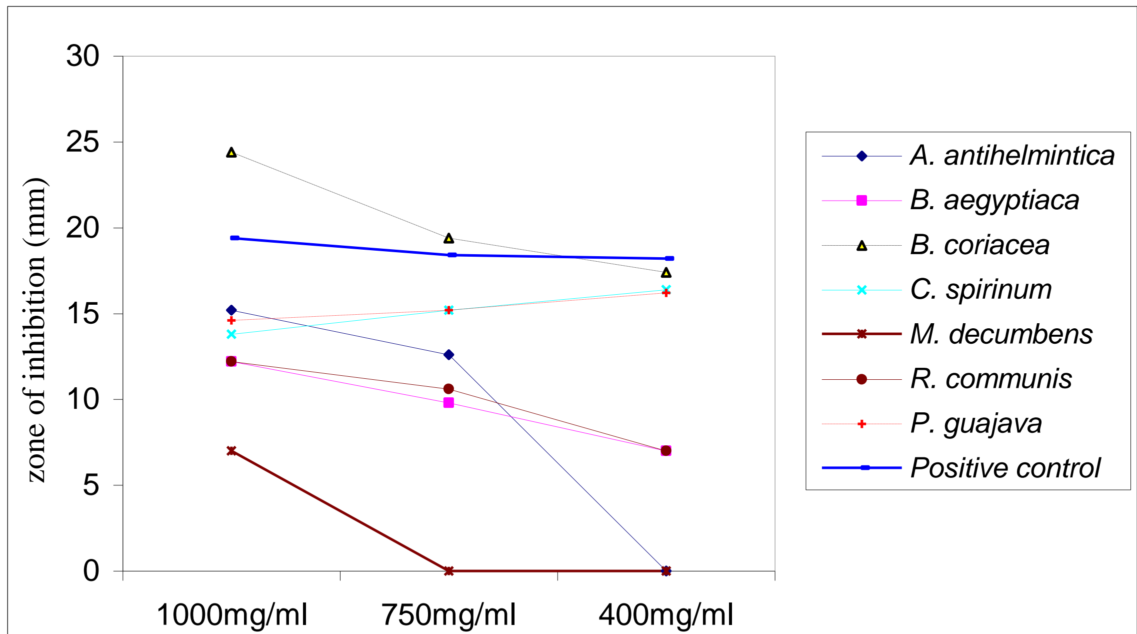


Figure 4.8a: Inhibition of growth of *A. flavus* by plant leaf extracts at the three concentrations

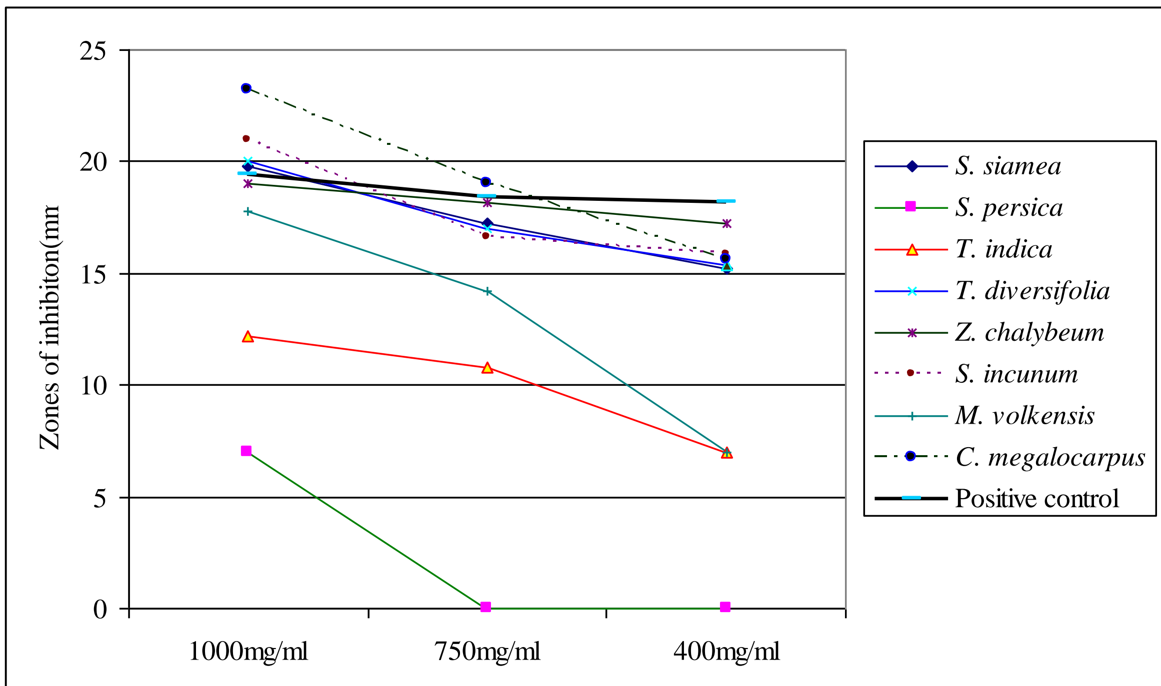


Figure 4.8b: Inhibition of growth of *A. flavus* by plant leaf extracts at the three concentrations

4.9.2 Efficacy of plant bark extracts on the growth of *A. flavus* at different concentrations

The results of inhibition zones of the bark extracts at a concentration of 1000 mg/ml indicated that there was a significant difference ($F = 159$, $df = 9$, $P < 0.05$) against the growth of the fungi. The exract of *S. siamea* (mean = 21.2 mm) had the highest inhibitory effect on *A. flavus* than the other bark extracts. The least inhibitory effect was detected with the bark extract of *S. incunum* (mean = 14.4 mm) (Table 4.10).

The results of inhibition zones of the plant bark extracts at a concentration of 750mg/ml ($F = 190.5$, $df = 9$, $P < 0.05$) revealed that, the extract of *S. siamea* (mean = 20.40 mm) was still most effective against growth of *A. flavus*. However the mean inhibition zone was slightly lower than at the 1000 mg/ml concentration level. The plant bark extracts with the least inhibitory effects were *T. diversifolia* (mean = 12.80 mm) and *S. incunum* (mean =13.00 mm) (Table 4.10).

The inhibition zones of the plant bark extracts at a concentration of 400 mg/ml showed that there was a significant difference ($F = 81.22$, $df = 9$, $P < 0.05$) in the inhibitory effects of the plants bark extracts. At this concentration the inhibitory effects by the plant extracts were lower than that of the positive control (mean =17.4 mm). The extract of C. *megalocarpus* (mean =15.00 mm) had the highest inhibition zone (Table 4.10).

The comparison of the inhibitory effects of the bark extracts against *A. flavus* at the three different concentrations used is shown in Figure 4.9.

Table 4.10: Efficacy of plant bark extracts on the growth of *A. flavus* at the different concentrations.

Plant extract	**Inhibition zone (means in mm) at 1000mg/ml**	**Inhibition zone (means in mm) at 750mg/ml**	**Inhibition zone (means in mm) at 400mg/ml**
B. coriacea	17.60a	14.20a	13.20a
C. spirinum	17.20a	16.40a	13.60a
C. megalocarpus	18.60a	16.40a	15.00a
S. siamea	21.20b	20.40b	13.00a
S. incunum	14.40c	13.00a	10.80b
T. diversifolia	14.80c	12.80a	13.00a
Z. chalybeum	17.80a	15.20a	14.00a
Positive control (10mg/mm)	17.60a	17.40a	17.40c
Negative control	0.00d	0.00c	0.00d

Note: Numbers are means of 10 replications. Means are separated by Turkeys Test. Numbers followed by the same letters in the same column are not significantly different (P < 0.05).

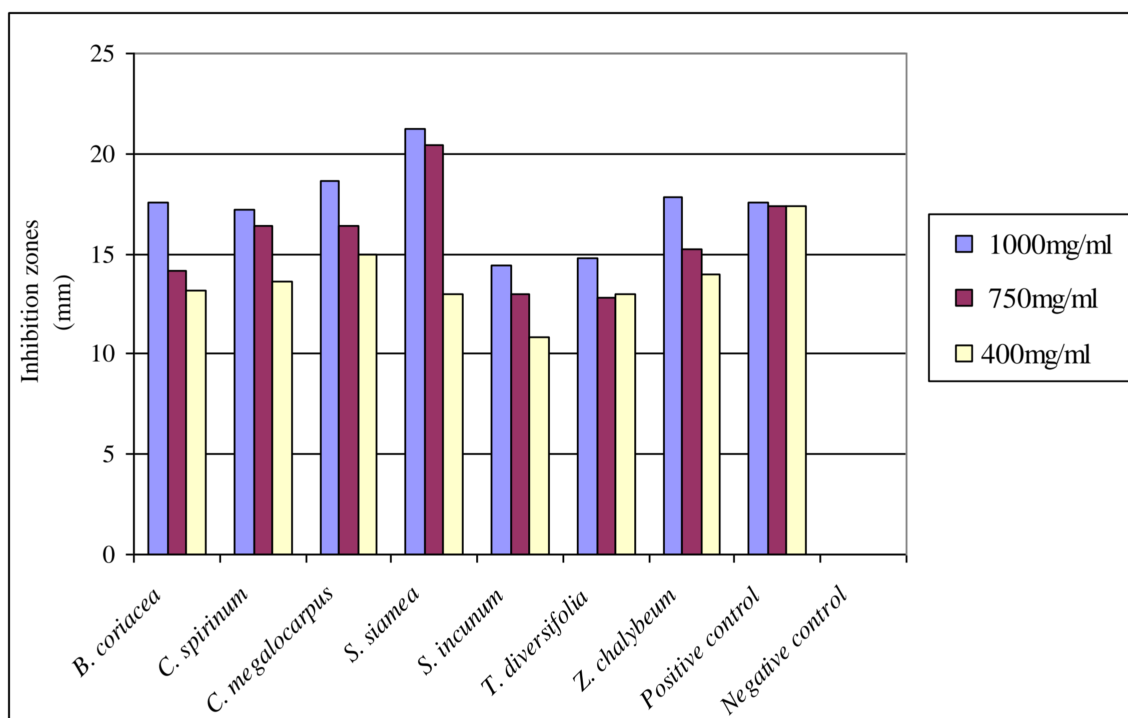


Figure 4.9: Inhibition of *A. flavus* by the plant bark extracts tested at the three different concentrations

The results of the inhibition zones of some of the plant extracts at the different concentrations are shown in plates 4.3a – 4.3f.

Plate 4.3a: Inhibition zones; *C. megalocarpus* 1000mg/ml

Plate 4.3b: Inhibition zones; *C. megalocarpus* 750mg/ml

Plate 4.3c: Inhibition zones; *T. diversifolia* 1000mg/ml

Plate 4.3d: Inhibition zones; *T. diversifolia* 750mg/ml

Plate 4.3e: Inhibition zones; *S. incunum* 750mg/ml

Plate 4.3f: Inhibition zones; *B. coriacea* 1000mg/ml

4.9.3 Determination of minimum inhibition concentrations (MIC) and minimum fungicidal concentrations (MFC) of leaf and bark extracts

The MIC and the MFC were done for the plant leaf and bark extracts that were found to be effective at 400 mg/ml.

4.9.4 The MIC and MFC of plant leaf extracts

Different plant extracts exhibited different MIC and MFC at the different concentrations used; 1.56mg, 3.125 mg. 6.25 mg, 12.50 mg and 25mg. The extracts of *B. coriace, S. incunum, Z. chalybeum, and S. siamea* recorded the lowest MIC and MFC of 6.25 mg/ml and 12.5 mg/ml respectively. This was followed by *C. spirinum, C. megalocarpus, T. diversifolia* and *M. volkensis* with MIC of 12.25 mg/ml. (Table 4.11).

4.9.5 The MIC and MFC of plant bark extracts

The MIC and the MFC of the bark extracts of the plants that proved to have inhibitory effects at a concentration of 400 mg/ml are shown in Table 4.12. The lowest MIC recorded was 6.25 mg/ml in the extracts of S. *siamea* and *Z. chalybeum.*

The results of the MIC and MFC of some of the plant extracts are shown in Plates 4.4a - 4.4f. In the negative control fungal growth occurred in all the four tubes (Plate 4.4 a). For the extracts of *B. coriacea,* the MIC was in the second tube and the MFC in the first tube while for *Z. chalybeum* the MIC was the in third tube and MFC were recorded in the second tube where no fungal growth was observed (Plate 4.4c).

Table 4.11: The MIC and MFC of plant leaf extracts (mg/ml)

Plant	MIC (mg/ml)		MFC (mg/ml)	
	Leaf	**Bark**	**Leaf**	**Bark**
B. coriacea	6.25	12.50	12.50	25.00
C. spirinum	12.50	12.50	25.00	12.50
C. megalocarpus	12.50	12.50	25.00	25.00
M. volkensis	12.50	12.50	12.50	25.00
S. siamea	6.25	6.25	12.50	12.50
T. diversifolia	12.50	12.50	25.00	25.00
Z. chalybeum	6.25	6.25	12.50	6.25
S. incunum	6.25	12.50	12.50	25.00
+VE Control Meconazole (10mg/ml)	10.00	10.00	10.00	10.00

Plate 4.4a: Negative control For MIC

Plate 4.4b:Tubes with SDB and leaf extracts of *B. coriacea*

Plate 4.4c: Tubes with SDB and leaf extracts of *Z .chalybeum*

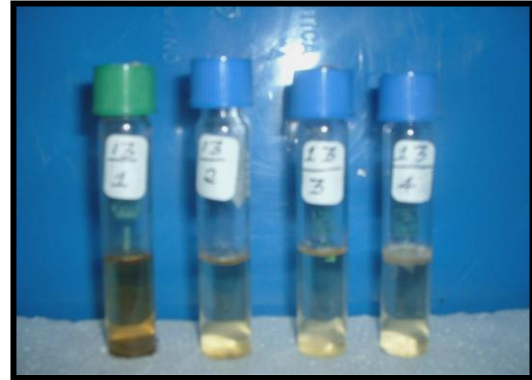


Plate 4.4d: Tubes with SDB and leaf extracts of *T. diversifolia*

Plate 4.4e: Tubes with SDB and leaf extracts of *C. megalocarpus*

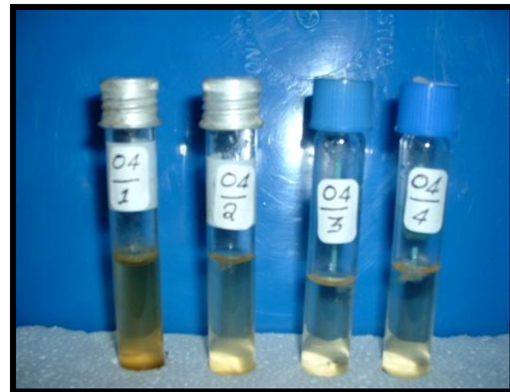


Plate 4.4f: Tubes with SDB and leaf extracts of *C. spirinum*

Key:

1 = 25 mg/ml; 2 = 12.5mg/ml; 3 = 6.25mg/ml; 4 = 3.125mg/ml

CHAPTER 5

DISCUSSION, CONLUSION AND RECOMMENDATIONS

5.1 Discussion

In this study, high incidences of *Aspergillus* sp. were found in the maize samples that were collected from the ten Divisions in the five Districts. The highest recorded incidence of *Aspergillus* sp. in maize in the study was 58.80%. The results of high incidences of *Aspergillus* sp. in this study agree with what has been previously reported in Nigeria in post-harvest maize (Bankole, 2003). It is likely that preharvest infections and the storage structures greatly influence the mycoflora in storage. The significantly high incidences of fungi in the *Aspergillus* sp. and the high aflatoxin levels elucidated in this study could possibly be attributed to some of the storage structures. Some of these structures used for maize storage in Eastern Kenya included woven polypropylene bags, heaping on the floor and on the verandah, above fire racks, and out-door storage practices like granaries.

According to Fandohan *et al.* (2005), some of these structures do not guarantee maize free from moisture pick-up, mould infection and hence protection of the grains against aflatoxin contamination. The high moisture content in some of the maize samples could also possibly have contributed to the high incidences of *Aspergillus* species. The moisture content of some of the maize samples collected from the farmers were found to be higher than 13% with some recording up to 16%. According to Abbas (2005), the fungi in the *Aspergillus* species can survive and grow in grains with moisture content as low as 15% while moisture levels below 12 to 13% inhibit growth of the fungus at any temperature.

There were four different species of *Aspergillus* isolated and identified from all the maize samples collected from the five Districts in this study. Although the distribution of members of *Aspergillus sp.* varied across the Divisions and the Districts, *A. flavus* was the most dominant species in most of the areas studied. The fungus recorded the highest icidences in three out of the five Districts and eight out of the ten Divisions studied. The fungus *A. tamarii* recorded the highest incidences in Kitui South and Machakos Districts.

The results of high incidences of *A. flavus* in maize from the same regions in Eastern Kenya agree with that of CDC (2004) and Probst *et al.* (2007). In an investigation on the causal agent of the 2004 outbreak of aflatoxicoses in the same region, large quantities of *A. flavus* were isolated from all maize samples that were evaluated (Probst *et al,* 2007). These investigations therefore support the high incidences of *A. flavus* to be attributed to the frequent outbreaks of aflatoxicoses in Eastern Kenya. Similar and high incidences of the fungus in stored maize have also been recorded in Benin (Atehnkeng *et al.,* 2008) and in three agroecological zones of Uganda (Kaaya and Warren, 2006). Although other fungi in the *Aspergillus* sp. infect maize in the field, *A. flavus* appears to be the dominant aflatoxin-producing fungus (Sétamou *et al.,* 1997), especially in tropical regions. As reported elsewhere, *A. flavus* is the most predominant member of *Aspergillus* in soils in West Africa and the United States (Atehnkeng *et al.,* 2008).

The fungus *A. flavus* is known for the production of several major mycotoxins that include aflatoxin B1, cyclopiazonic acid (CPA), and 3- nitropropionic acid. The fungus, *A. tamarii* has been reported to produce various mycotoxins that include CPA, fumigaclavine A, and kojic acid (Klich, 2002). This fungus is also recognized as a colonist of seeds and causes

aflatoxin contamination in cotton seeds, peanuts, pistachio nuts, sun- flower seeds, corn grains and rice grains (Goto *et al*., 1996). Single-spore isolates of *A. tamarii* have been reported to produce aflatoxin B1 and B2 in glucose yeast extract (GY) liquid medium. This now groups *A. tamarii* as one of the aflatoxin-producing species together with *A. flavus*, *A. parasiticus*, and *A.nomius* (Goto *et al*., 1996). From our results, it is possible that contamination of maize with aflatoxin B1 in Eastern Kenya could be attributed to *A. flavus* and *A. tamari.* The fungus, *A. ochraceous* is also known to produce various mycotoxins that include penicillinic acid, ochratoxin A, xanthomeginin, viomellein and vioxanthin. The fungi *A. niger* produces Ochratoxin A (Klich, 2002). The occurrence of *A. niger*, *A. ochraceous* and *A. tamarii* in maize in Eastern Kenya has not been adequately reported. Due to the presence the *A. niger*, *A. ochraceous* and *A. tamarii* in the maize from Eastern Province of Kenya, it is probable that maize in these region is contaminated with other mycotoxins apart from aflatoxin B1.

In our study, the maize samples that were analysed for aflatoxin were found to contain different levels of aflatoxin B1. Of the 10 samples analysed for aflatoxin levels, 5 contained > 20ppb of aflatoxin while the other 5 recorded < 20ppb of aflatoxin B1. This type of aflatoxin found in the maize samples in the study, is considered the most toxic aflatoxin. The potency of aflatoxin B1 when it contaminates food grains is supported by Lanyasunya *et al*. (2005). Its potency was illustrated by an outbreak of lethal aflatoxicoses that took more than 125 lives in Eastern Province of Kenya between January and July 2004 (Probst *et al.,* 2007).

It has been noted that chronic low-level aflatoxin exposure can increase the risk for hepatocellular carcinoma (Peraica *et. al.,* 1999). According to Chao e*t al.* (1991) high-dose exposures to aflatoxins leads to severe, acute liver injury with high morbidity and mortality. Ingestion of 2 - 6 mg/day of aflatoxin for a month can cause acute hepatitis and death. Therefore cumulative levels of aflatoxin B1 could cause health problems to people in Eastern province of Kenya. Similar reports have associated aflatoxin B1 with acute hepatitis caused by the consumption of moldy grains reported in other areas in Africa, Western India, and Malaysia (Lanyasunya *et al*., 2005) where most affected persons came from areas prone to drought and malnutrition. The Eastern Province of Kenya is characterized by abiotic factors marked by increasing temperatures, decreasing moisture and low and unreliable rainfall. Besides the unpredictable change in rainfall pattern sometimes causes forced harvest of grains before adequate drying, leading to heavy colonization of stored maize by the *Aspergillus sp.* and consequently aflatoxin production.

Knowing the hazards of aflatoxin B1 exposure, the need for protection of foods and feedstuffs against aflatoxin is universally recognized and several approaches have been suggested. The FDA and WHO have established an acceptance level of 20 ppb for aflatoxin in maize for human consumption (Grybauskas *et al.,* 2000). Maize containing aflatoxin levels greater than 20 ppb or more should thus not be consumed by humans (Grybauskas *et al.,* 2000).

The study established that there was negative correlation between the incidences of *A. flavus* and aflatoxin levels ($r = -0.758$, $P = 0.01$) in some Divisions such as Kathiani,

Kibwezi, Mutomo and Makindu. This could possibly be due to the presence of the highly toxigenic S strain of *A. flavus.* This strain is attributed with production of relatively high levels of aflatoxins (Bennett and Klich, 2004). There were also negative correlation of high incidences of *A. flavus* and low aflatoxin levels in some other Divisions such as Matungulu, Kangundo, Kaiti and Mutha. This could possibly be due to the presence of the atoxigenic L strain of *A. flavus* which is attributed with production of low levels of aflatoxins (Bennett and Klich, 2004).

This study was also undertaken to evaluate the antifungal activity of extracts from 15 selected medicinal plants. The preliminary phytochemical screening revealed the presence of various bioactive agents. These agents included tannins, flavonoids, terpenoids, cardiac glycosides, saponins and alkaloids. This agrees with reports by Parekh and Chanda, (2007) and Ramkumar *et al.* (2007) that the antifungal activity of the plant extracts is mainly due to the presence of flavanoids, steroids, alkaloids and triterpenoids and other natural polyphenolic compounds or free hydroxyl groups. In this study the preliminary phytochemical analysis revealed the presence of these compounds in the various plant extracts used and hence the efficacy of the extracts against *A. flavus*.

Both the methanolic leaf and bark extracts of the plants assayed displayed concentration dependent antifungal activities that were comparable to that of the reference drug Meconazole at 10 mg/ml (Tables 4.19 and 4.26). Out of the 15 plant leaf extracts, thirteen indicated significant ($P<0.05$) antifungal activity at 1000 mg/ml and and eleven indicated significant ($P<0.05$) antifungal activity at 750 mg/ml. This activity was higher than that of

the reference antifungal drug Meconazole at 10 mg/ml. At a concentration of 400 mg/ml, eight plant axtracts inhibited growth of *A. flavus* and also showed large inhibition zones against the fungus. The results of this study confirm the efficacy of these plants against microorganisms and their traditional use in food storage. The *in vitro* activities of the crude extracts of these plants also provides evidence to support the use of such plants in the control of *A. flavus* (Hassan *et al.,* 2006)

The extracts of *B. coriacea* had a good efficacy against *A. flavus* and this is in agreement with results of Hassan *et al.,* (2006) who reported on compounds with antimicrobial activity in the plant. In our study, the results of phytochemical screening indicated the presence of alkaloids, terpenoids and flavonoids in the plant extracts. Therefore, possibly these compounds may be responsible for the antifungal activity of the plant extracts of *B. coriacea* and hence the high efficacy against *A. flavus.*

The results of the efficacy of *C. megalocarpus* against *A. flavus* are attributed to the presence of terpenoids and tannins and flavonoids (Fontenelle *et al.,* 2008). In our study, the preliminary phytochemical screenings of the plant extract showed the presence of terpenoids and alkaloids that have been found to have antifungal activity. The efficacy of *C. megalocarpus* against *A. flavus* agrees with results of Fontenelle *et al*. (2008) who found these compounds in other *Croton sp.* to have antifungal activities. The bioactive agents from *C. nepetaefolius*, *C. argyrophylloides, C. zenhteneri* and *C. Tehntneri* were shown to have antifungal activity against *A. parasiticus* and *Candida* species. The results of our study are in agreement with Pyun and Shin, (2006), that these bioactive agents in *Croton*

sp. could be one of the most promising compounds from which a new prototype of antifungal agents can be developed.

The efficacy of *C. spinirum* (*C. edulis*) against *A. flavus* agree with the results by Festus *et al.* (2007) who found the leaves of the plant to contain tannins and saponnins that have been reported to have antimicrobial activity. In our study, the extract of this plant showed the presence of these two compounds after carrying out phytochemical screening. Our results are also in agreement with that of Ibrahim *et al.* (2005) who found the leaf extracts of the the plant to be effective against *C. albicans* and attributed this to the presence of tannins or saponins. Therefore the results justify the use of the leaves in ethnomedicine.

The results of our antifungal activity of *S. siamea* (*Cassia siamea*) against *A. flavus* have been supported by previous reports of Phongpaichit *et al.* (2004) of its antimicrobial activities against human pathogens. The results they obtained from a study using leaf extracts of a plant in the same Genus; *S. alata,* showed antifungal activity against some dermatophytes and yeast (*Trichophyton rubrum, Microsporum gypseum* and *Penicillium marneffei*) (Phongpaichit*, et al.,* 2004). The antifungal activity of *S. siamea* against *A. flavus* in our study, is in agreement with the results of Palanich-amy and Nagarajan, (1990) who found the leaf extracts of *S. alata,* to possess antifungal activity against *A. flavus, C. albicans, Aspergillus fumigatus, Mucor* sp., *Rhizopus* spp. and dermatophytes; *Trichophyton mentagrophytes, T. rubrum* and *M. gypseum* (Phongpaichit *et al.,* 2004). According to Duraipandiyan *et al*. (2006**),** whose results from crude flavonoid extract from the flowers *S. siamea* illustrated better microbial activity; the efficacy of this plant is attributed to the presence of flavonoids. This is in agreement with our results that showed

the presence of flavonoids from the extracts of *S. siamea* in the preliminary phytochemical screening.

In our study, the extracts of *Z. chalybeum* showed a good efficacy against *A. flavus* and this agrees with results of Ngono *et al*. (2000) who worked on two other species of *Zanthoxylem; Z. leprenirri and Z. xantholoides* and found that they possesed antifungal activities. According to Adesina (2005), the antifungal activity of this plant may be due to the presence of various metabolites that include alkaloids. The results our study showed the presence alkaloids, terpenoids and flavonoids in the extracts of *Z. chalybeum*. Adesina (2005) also found metabolites isolated from *Z. zanthoxyloides,* which include alkaloids, aliphatic and aromatic amides, lignans, coumarins, sterols, carbohydrate residues etc. to have antimicrobial activities. Another compound with antimicrobial activity in this plant is Zanthoxylol and it has been isolated and estimated from the roots of the only Nigerian *Zanthoxylem* in which it was detected (Adesina, 2005).

The plant extracts found to have high antifungal effects recorded the lowest MICs and MFCs. Both the leaf and bark extracts of *Z. chalybeum* and *S. siamea* had low MIC of 6.25 mg/ml. This possibly has enhanced high antifungal activivties of the plant extracts. The other plants such as *C. spinirum, C. megalocarpus* and *B. coriacea* had relatively low MIC OF 12.5 mg/ml and these could also have enhanced their efficacy agaist *A.flavus.* The study also revealed that the plant extracts tested had MFCs that were higher than the MICs.

5.2 Conclusion

The results of high incidences of *A. flavus* and high levels of aflatoxin B1 in our study using stored maize from Makueni, Machakos, Kitui South, Kangundo and Kibwezi Districts confirms *A. flavus* as the possible causal agent of the frequent aflatoxicoses in Eastern Kenya. It also possibly explains the cause of the lethal aflatoxicoses that claimed more than 125 lives in 2004 in Eastern and Central Provinces of Kenya. Besides *A. flavus* our study has revealed the presence of other three *Aspergillus* species that include *A. niger*, *A. ochraceous* and *A. tamarii* in the maize samples collected from Eastern Kenya. These fungi are known to produce various mycotoxins that have proved to be hazardous to human health. Therefore similar efforts given to the study on *A. flavus* and aflatoxins should also be directed towards these three *Aspergillus* species and the effects of their mycotoxins on food contamination and to human health.

High incidences of *A. flavus* in stored maize in Eastern Province of Kenya indicate high levels of aflatoxin contamination. It is uncontestable fact that aflatoxins pose a serious health risk to both livestock and human beings world-over, particularly in the tropics where climatic conditions (high moisture and temperature) spur the growth of moulds. The Eastern Province of Kenya as a case in point has encountered frequent outbreaks of aflatoxicoses. There is no cure for aflatoxin B1 poisoning and it damages vital organs in the body including the liver, kidneys and lungs. Other than the direct health risk, economic losses arising from aflatoxicoses are equally enormous. Therefore controlling mould growth and aflatoxin production is very important. First step in mould control is to ensure that the maize grains are dried adequately. The dried grains should then be stored in well-

aerated storage structures and at low moisture level (14 percent or less) to discourage mould growth and mycotoxin contamination.

Antifungal chemicals have been used for the preservation of stored grains. Due to health and economic considerations, natural plant extracts may provide an alternative method to protect stored maize from fungal contamination. Recently much attention has been directed towards extracts and biologically active compounds isolated from popular plants species. In recent years the popularity of complementary medicine has increased. The World Health Organization has also recommended the evaluation of the plants effective in conditions where safe modern drugs are lacking (Ramkumar *et al.,* 2007). This has led to plant substances serving as viable sources of drugs for the world population and several plant-based drugs are in extensive clinical use. The use of medicinal plants plays a vital role in covering the basic health needs in the developing countries and these plants may offer a new source of antibacterial, antifungal and antiviral agents with significant activity against microorganisms (Leite *et al.,* 2006). This has been evoked by the increasing development of drug resistance in human pathogens as well as the appearance of undesirable effects of certain antifungal agents (Phongpaichit *et al.,* 2005) hence a call for a need to search for new antimicrobial agents.

Therefore there is an absolute need for bioactivity guided fractionation and isolation of the active components for the plant extracts found to be effective against *A. flavus.* This study evaluated fifteen medicinal plants and eight were found to have a high efficacy against *A. flavus.* The eight plants contained effective phytochemical compounds that could be employed as inhibitors of fungal growth and aflatoxin production in the storage of maize

and other cereal grains. The antifungal properties of *B. coriacea*, *Z. chalybeum*, *C. spirinum*, *S. siamea, S. incunum, C. megalocarpus* and *T. diversifolia* indicates that these plants are potential candidates for alternative management practices against *A. flavus* and aflatoxin production in the storage of maize and other cereal grains. The study also highlights the need to screen more plants as they may also contain the effective bioactive compounds in large quantities as it has been shown by the medicinal plants collected from Mwingi District in Kenya.

5.3 Recommendations

- Storage systems creating unfavourable conditions for fungal growth and aflatoxin production are therefore recommended.
- Sorting out of damaged cobs or grains at harvest and choice of storage structures that ensure good drying of maize are recommended to farmers in Eastern Province of Kenya.
- Enhanced efforts should be geared towards the prevalence of other storage fungi in the *Aspergillus* species as they may also be a health hazard to the people living in these regions.
- To identify the methods of usage of these plant extracts which may be dried, powdered, concentrated and then mixed with stored maize grains.
- Further studies need to be carried out for bioactivity guided fractionation and isolation of the active components of the plant extracts found to have antifungal properties. This may lead to elucidation of new and effective antifungal agents that may serve as an alternative management strategy of *A. flavus* and other storage fungi in maize and other cereal grains.

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APPENDIX

Appendix 1

Modified Rose Bengal Agar (MRBA)

Media prepared by combining the following per liter of distilled water;

Inorganic salts that included

$NaNO_3$	3.0g
KH_2PO_4	0.3g
K_2HPO_4	0.7g
$MgSO_4.7\ H_2O$	0.5g
KCl.	0.5g
NaCl	10.0g
Bacto agar	20.0g

Micronutrients of Adye and Mateles

$Na_2B_4O_7.\ 10H_2O$	0.7mg
$(NH_4)\ _6MO_7O_{24}.\ 4H_2O$	0.5mg
$Fe\ (SO_4)_3.6H_2O$	10.0mg
$CuSO_4.5H_2O$	0.3mg
$MnSO_4.H_2O$	0.11mg
$ZnSO_4.7H_2O$	17.5mg

Antibiotics

Chloramphenical	50.0mg
Dicholran	10.0mg
Streptomycin	0.3g
Rose Bengal Stock	5ml
Sucrose	3.0g

Appendix 2

Czapeck Yeast Agar (CYA25)

Media prepared by combining the following per liter of distilled water:

$NaNO_3$	30.0g
$MgSO_4.7H_2O$	5.0g
$Fe\ (SO_4)_3.7H_2O$	0.1g
$ZnSO_4.7H_2O$	0.1g
$CuSO_4.5H_2O$	0.5g

The micronutrients are dissolved in 100ml distilled water,

K_2HPO_4	1.0g
Powdered Yeast Extract	5.0g
Sucrose	30.0g
Agar	15.0g

Appendix 3

Table 6.1: Maximum acceptable levels for aflatoxin for a selection of countries (Aflatoxin B, unless otherwise stated)(FAO., 2002)

Country	Limit (µg kg^{-1})	Foods
United Kingdom	2	Nuts, dried figs and their products
	5	As above but intended for further processing
United States	20	Total aflatoxins in all foods
	0.5	Aflatoxins M_1 in wholemilk,lowfat Milk and skim milk
Australia	5	All foods except peanut products
	15	Peanut products
India	30	All foods
Japan	10	All foods
China	50	Rice, peanuts, maize, sorghum, beans, Wheat, barley, oats.

Appendix 4: Acceptance level for aflatoxin (FDA, 2002)

Mycotoxin	***FDA tolerance***	***Commodity and/or use***
Aflatoxin M_1	0.5 ppb	Milk
Aflatoxin	20 ppb	Corn and other animal feeds and feed ingredients, excluding cottonseed meal, intended for immature animals, Corn, cottonseed meal, and other animal feed ingredients intended for dairy animals, for animal species or uses not specified above, or when intended use is not known Foods (for humans)
	100 ppb	Corn intended for breeding beef cattle, breeding swine, or mature poultry
	200 ppb	Corn intended for finishing swine of 100 lbs or greater
	300 ppb	Corn intended for finishing (i.e. feedlot) cattle

Appendix 5

One-way ANOVA: Total *Aspergillus sp*. incidence versus the 5 District

Analysis of Variance for fungi

Source	DF	SS	MS	F	P
District	4	8462	2115	6.13	0.000
Error	45	15519	345		
Total	49	23981			

Appendix 6

One-way ANOVA: Total *Aspergillus sp*. incidence versus the 10 Divisions

Analysis of Variance for fungi

Source	DF	SS	MS	F	P
Division	9	9188	1021	2.76	0.013
Error	40	14794	370		
Total	49	23981			

Appendix 7

One-way ANOVA: *Aspergillus* sp. incidence versus Kangundo District.

Analysis of Variance for Kangundo

Source	DF	SS	MS	F	P
Aspergillus	3	87.748	29.249	33.78	0.000
Error	36	31.171	0.866		
Total	39	118.919			

Appendix 8

One-way ANOVA: *Aspergillus sp.* incidence versus Kibwezi District.

Analysis of Variance for Kibwezi

Source	DF	SS	MS	F	P
Aspergillus	3	50.401	16.800	26.06	0.000
Error	36	23.212	0.645		
Total	39	73.613			

Appendix 9

One-way ANOVA: *Aspergillus sp.* incidence versus Makueni District.

Analysis of Variance for Makueni

Source	DF	SS	MS	F	P
Aspergillus	3	56.467	18.822	27.29	0.000
Error	36	24.829	0.690		
Total	39	81.296			

Appendix 10

One-way ANOVA: *Aspergillus sp.* incidence versus Kitui South District.

Analysis of Variance for Kitui South

Source	DF	SS	MS	F	P
Aspergillus	3	116.96	38.99	21.78	0.000
Error	36	64.46	1.79		
Total	39	181.42			

Appendix 11

One-way ANOVA: *Aspergillus sp.* incidence versus Machokos District.

Analysis of Variance for Machokos

Source	DF	SS	MS	F	P
Aspergillus	3	48.66	16.22	12.14	0.000
Error	36	48.11	1.34		
Total	39	96.76			

Appendix 12

One-way ANOVA: *Aspergillus sp.* incidence versus Kangundo Division

Analysis of Variance for kangundo

Source	DF	SS	MS	F	P
Aspergillus	3	34.97	11.66	11.51	0.000
Error	16	16.20	1.01		
Total	19	51.17			

Appendix 13

One-way ANOVA: *Aspergillus sp.* incidence versus Matungulu Division

Analysis of Variance for matungulu

Source	DF	SS	MS	F	P
Aspergillus	3	54.106	18.035	21.41	0.000
Error	16	13.477	0.842		
Total	19	67.583			

Appendix 14
One-way ANOVA: *Aspergillus sp.* incidence versus Kibwezi Division

Analysis of Variance for Kibwezi

Source	DF	SS	MS	F	P
Aspergillus	3	23.359	7.786	24.47	0.000
Error	16	5.092	0.318		
Total	19	28.451			

Appendix 15

One-way ANOVA: *Aspergillus sp.* incidence versus Makindu Division.

Analysis of Variance for Makindu

Source	DF	SS	MS	F	P
Aspergillus	3	27.750	9.250	9.43	0.001
Error	16	15.690	0.981		
Total	19	43.440			

Appendix 16

One-way ANOVA: *Aspergillus sp.* incidence versus Wote Division

Analysis of Variance for Wote

Source	DF	SS	MS	F	P
Aspergillus	3	16.125	5.375	16.70	0.000
Error	16	5.150	0.322		
Total	19	21.276			

Appendix 17

One-way ANOVA: *Aspergillus sp.* incidence versus Kaiti Division.

Analysis of Variance for Kaiti

Source	DF	SS	MS	F	P
Aspergillus	3	49.616	16.539	27.39	0.000
Error	16	9.662	0.604		
Total	19	59.277			

Appendix 18

One-way ANOVA: *Aspergillus sp.* incidence versus Machakos Central Division.

Analysis of Variance for Central

Source	DF	SS	MS	F	P
Aspergillus	3	18.698	6.233	12.03	0.000
Error	16	8.293	0.518		
Total	19	26.991			

Appendix 19

One-way ANOVA: *Aspergillus sp.* incidence versus Kathiani Division.

Analysis of Variance for Kathiani

Source	DF	SS	MS	F	P
Aspergillus	3	40.66	13.55	8.12	0.002
Error	16	26.72	1.67		
Total	19	67.37			

Appendix 20

One-way ANOVA: *Aspergillus sp.* incidence versus Mutomo Division.

Analysis of Variance for Mutomo

Source	DF	SS	MS	F	P
Aspergillus	3	76.51	25.50	8.99	0.001
Error	16	45.38	2.84		
Total	19	121.89			

Appendix 21

One-way ANOVA: *Aspergillus sp.* incidence versus Mutha Division.

Analysis of Variance for Mutha

Source	DF	SS	MS	F	P
Aspergillus	3	45.519	15.173	26.46	0.000
Error	16	9.174	0.573		
Total	19	54.693			

Appendix 22

One-way ANOVA: *A. flavus* incidence versus Districts

Analysis of Variance for *A. flavus*

Source	DF	SS	MS	F	P
District	4	27.596	6.899	7.73	0.000
Error	45	40.186	0.893		
Total	49	67.781			

Appendix 23

One-way ANOVA: *A. flavus* incidence versus Divisions

Analysis of Variance for *A. flavus*

Source	DF	SS	MS	F	P
All divisions	9	35.563	3.951	4.91	0.000
Error	40	32.219	0.805		
Total	49	67.781			

Appendix 24

One-way ANOVA: *A. niger* incidence versus Divisions

Analysis of Variance for *A. niger*

Source	DF	SS	MS	F	P
All Divisions	9	35.088	3.899	3.97	0.001
Error	40	39.241	0.981		
Total	49	74.329			

Appendix 25

One-way ANOVA: *A. ochraceous* versus Divisions

Analysis of Variance for *A. ochraceous*

Source	DF	SS	MS	F	P
All Divisions	9	25.908	2.879	6.00	0.000
Error	40	19.186	0.480		
Total	49	45.094			

Appendix 26

One-way ANOVA: *A. tamarii* versus Divisions

Analysis of Variance for *A. tamarii*

Source	DF	SS	MS	F	P
All Divisions	9	145.39	16.15	10.15	0.000
Error	40	63.66	1.59		
Total	49	209.05			

Appendix 27

One-way ANOVA: Inhibition (mm) zones of *A. flavus* versus 1000mg/ml plant leaf extracts

Analysis of Variance for inhibition

Source	DF	SS	MS	F	P
Plants	15	2282.59	152.17	27.30	0.000
Error	64	356.80	5.58		
Total	79	2639.39			

Appendix 28

One-way ANOVA: Inhibition zones (mm) of *A. flavus* versus 750mg/ml plant leaf extracts

Analysis of Variance for inhibition

Source	DF	SS	MS	F	P
Plants	15	2875.29	191.69	94.08	0.000
Error	64	130.40	2.04		
Total	79	3005.69			

Appendix 29

One-way ANOVA: Inhibition zones (mm) of *A. flavus* versus 400mg/ml plant leaf extracts

Analysis of Variance for inhibition

Source	DF	SS	MS	F	P
Plants	15	3653.40	243.56	100.70	0.000
Error	64	154.80	2.42		
Total	79	3808.20			

Appendix 30

One-way ANOVA: Inhibition at 1000mg/ml versus plants

Analysis of Variance for inhibition

Source	DF	SS	MS	F	P
Plants	8	1460.80	182.60	144.79	0.000
Error	36	45.40	1.26		
Total	44	1506.20			

Appendix 31

One-way ANOVA: Inhibition zones at 750 versus plants

Analysis of Variance for inhibition

Source	DF	SS	MS	F	P
Plants	8	1319.778	164.972	196.66	0.000
Error	36	30.200	0.839		
Total	44	1349.978			

Appendix 32

One-way ANOVA: Inhibition zones at 400 versus plants

Analysis of Variance for inhibition

Source	DF	SS	MS	F	P
Plants	8	965.78	120.72	92.47	0.000
Error	36	47.00	1.31		
Total	44	1012.78			