AFLATOXIN LEVELS IN SUNFLOWER SEEDS, CAKES, AND CRUDE OIL SEDIMENTS LOCALLY PRODUCED IN TANZANIA AND POTENTIAL PHYTOCHEMICALS FOR AFLATOXIN MANAGEMENT

By

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ABSTRACT

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This dissertation had two objectives. The first was to determine total aflatoxin concentrations in sunflower seeds, cakes and crude oil sediments from small-scale oil processors across Tanzania. The towns selected for sunflower sample collection included Mbeya, Iringa, Morogoro, Dodoma, Singida, Babati-Manyara and Karatu-Arusha. We collected a total of 232 samples: sunflower seed (n = 90), cake samples (n = 92) and crude sunflower oil sediments (n = 50) across two years, which were analyzed for total aflatoxin concentrations using a direct competitive enzyme-linked immunosorbent assay (ELISA). The aflatoxin surveillance performed in June-August 2014, indicated that the highest aflatoxin levels in sunflower seeds were from the towns of Babati-Manyara, Singida, and Dodoma. The aflatoxin levels in sunflower cakes were exceedingly high in Singida, Dodoma, and Mbeya. The surveillance performed in August-October 2015, indicated that the highest aflatoxin concentrations in sunflower seeds were from Mbeya, Singida, and Morogoro. Singida, Dodoma, and Morogoro had shown unacceptable aflatoxin levels in sunflower cakes. Aflatoxin levels in oil sediments were considerably lower in both years except Morogoro, which showed two oil sediments exceedingly contaminated (41.7 and 85.3 ng/g). Concerning risk assessment, Dodoma and Babati-Manyara showed dietary exposures of 25 and 21 ng/kg bw/day, respectively, for sunflower seed consumption in 2014. Liver cancer risks for these exposures were 0.9 and 0.8 cases per year per 100,000 individuals, respectively. Samples from Morogoro suggested dietary exposure of 24 ng/kg bw/day for sunflower seed consumption

in 2015 that carried a risk of 0.9 cases per year per 100,000 individuals. Although the crude oil sediment data showed that crude oils were safe, the general results of aflatoxin levels in seeds and cakes particularly from Manyara, Singida, Dodoma and Morogoro, and crude oil sediments from Morogoro, indicate that there is a potential risk of exposure to aflatoxin through sunflower products and intervention strategies are required.

Our second objective was to determine the anti-aflatoxigenic properties of compounds from a traditional medicinal plant *D. mafiensis* root bark against vegetative growth, sporulation and aflatoxin production by *Aspergillus flavus* and *Aspergillus parasiticus*. The bioactive compounds diosquinone (DQ) and 3-hydroxydiosquinone (3HDQ) were elucidated and identified using ^{1}H- and ^{13}C-NMR and LC-MS methods. Growth inhibition was determined by measuring the colony diameters of the molds in culture. Total aflatoxin was quantified by direct competitive enzyme-linked immunosorbent assay (ELISA). DQ showed weak potency against *A. flavus* and *A. parasiticus* vegetative growth (MIC_{50} >100 µg/mL), and 3HDQ demonstrated a strong potency against *A. flavus* (MIC_{50} = 14.9 µg/mL) and *A. parasiticus* (MIC_{50} = 39.1 µg/mL). Despite its weak potency against vegetative growth, DQ strongly reduced total aflatoxin production by *A. flavus* and *A. parasiticus* for over 90 %. Counterintuitively, 3HDQ stimulated aflatoxin production by *A. flavus* at lower doses but started to reduce aflatoxin production at the dose of 100 µg/mL. 3HDQ strongly reduced total aflatoxin production by *A. parasiticus* even at lower doses. In summary, DQ and 3HDQ could be used as natural fungicides to prevent mold growth and aflatoxin accumulation in food and feed.

This dissertation is dedicated to my late parents Abdallah Mmongoyo and Idaya Ching'ang'a. It takes great parents to raise a child. I am forever indebted to them.

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postdoctoral fellows added value to my scientific growth in the area of bioactive natural product chemistry.

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KEY TO ABBREVIATIONS

A.	*Aspergillus*
a.m.u	Atomic Mass Unit
ABPA	Allergic Brochopulmonary Aspergillosis
AFB_1	Aflatoxin B_1
AFB_2	Aflatoxin B_2
AFG_1	Aflatoxin G_1
AFG_2	Aflatoxin G_2
AFM_1	Aflatoxin M_1
AFP_1	Aflatoxin P_1
AFQ_1	Aflatoxin Q_1
ANOVA	Analysis of Variance
AOAC	Association of Official Agricultural Chemists
AVN	Averantin
CFU	Colony Forming Unit
CSC	Central Sunflower Corridor
CYP450	Cytochrome P450
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic Acid
DQ	Diosquinone
ELISA	Enzyme-linked Immunosorbent Assay

ESIMS	Electrospray Ionization Mass Spectrometry
FAO	Food and Agricultural Organization
FDA	Food and Drug Administration
GIT	Gastrointestinal Tract
GMS	Glucose Minimal Salts
GST	Glutathione S-transferase
GTP	Green Tea Polyphenols
HBV	Hepatitis B Virus
HCC	Hepatocellular Carcinoma
HCV	Hepatitis C Virus
HPLC	High Performance Liquid Chromatography
HRESIMS	High Resolution Electrospray Ionization Mass Spectrometry
^{13}CNMR	Carbon Nuclear Magnetic Resonance
^{1}HNMR	Proton Nuclear Magnetic Resonance
3HDQ	3-Hydroxydiosquinone
IARC	International Agency for Research on Cancer
IPM	Integrated Pest Management
JECFA	Joint FAO/ WHO Expert Committee on Food Additive
kb	kilobase
LC-MS	Liquid Chromatography –Mass Spectrometry
MAFSC	Ministry of Agriculture, Food Security and Cooperatives
MIC_{50}	50% Minimum Inhibitory Concentration
MPLC	Minimum Pressure Liquid Chromatography

NA	Norsolorinic Acid
OH	Hydroxyl group
PCR	Polymerase Chain Reaction
PDA	Potato Dextrose Agar
PKS	Polyketide Synthase
ROS	Reactive Oxygen Species
SEM	Scanning Electron Microscopy
Ser	Serine
SG	Glutathione
ST	Sterigmatocystin
TBS	Tanzania Bureau of Standards
TFDA	Tanzania Food and Drug Authority
TLC	Thin-Layer Chromatography
TMA	Tanzania Meteorological Authority
TP53	Tumor Protein 53
WHO	World Health Organization

INTRODUCTION

Aflatoxins are very potent food and feed contaminants naturally produced as polyketide-derived secondary metabolites by the fungi *Aspergillus flavus* and *A. parasiticus*. These contaminants frequently occur on cereals and oilseeds, and they have deleterious health effects on humans and animals exposed to them primarily through contaminated food and feed.

About 4.5 billion people are exposed to aflatoxin through contaminated food worldwide each year (Strosnider et al. 2006). This problem of chronic exposure of individuals to aflatoxin is directly linked to incidence of aflatoxin-induced liver cancer deaths worldwide. The WHO (2008) statistics show that between 125,000 and 155,000 people die of aflatoxin-induced liver cancer yearly worldwide, the greatest mortality rate being predominant in Eastern Asia and Sub-Saharan (Liu and Wu, 2010). It is alarming that the communities with a high incidence of chronic hepatitis B virus (HBV) and hepatitis C virus (HCV) infections are 30 times more at risk of aflatoxin-induced liver cancer than those without (Groopman et al. 2005). Perhaps, this is because the virus attacks and compromises the aflatoxin detoxification ability of the liver (Groopman et al. 2005). Domestic animals such as dairy cows, poultry and swine are also vulnerable to dietary exposure to aflatoxin through contaminated feedstuffs leading to underweight and illness in animals.

In tropical low-income countries, much of production and accumulation of aflatoxins in food and feed is more severe during post-harvest storage because of poor storage conditions favorable to profuse mold growth and aflatoxin production. The post-harvest contamination of food crops does not only increase the risk of exposure to humans and animals to aflatoxin, but the fungal deterioration of these crops causes significant agricultural and economic losses. For

example, the USA alone loses nearly US$ 500 million annually due to disposal of aflatoxin-contaminated maize, peanuts, and cottonseeds; and underweight animals fed with contaminated feedstuffs (Vardon et al. 2003). The stringent FDA food and feed regulations intended to protect the public and animals from deleterious effects of aflatoxin also impose enormous losses because food and feed with aflatoxin levels exceeding the regulatory guidelines are deemed unsuitable for human and animal consumption and they are discarded or sold at lower prices for other uses (Wu et al., 2008). On the contrary, food and feed in low-income countries are rarely regulated for aflatoxin levels, as food laws enforcement in such countries is currently impractical due to lack of infrastructure, technology, and routine aflatoxin monitoring as the crops are mainly homegrown by smallholder farmers (Wu 2004). Sadly, when farmers want to obtain cash from portions of their maize, peanuts, sunflower, and sesame harvests, they find themselves sell high-quality seeds to the traders and keep poor-quality seeds for food and feed thus, exacerbating their exposure to aflatoxins (Wu 2004). Because severe aflatoxin contamination of crops in the low-income countries occurs in storage, it would be imperative to assess aflatoxin levels on a regular basis. Also, it is critical to search for antifungal strategies to reduce the aflatoxin production on crops under storage conditions.

Sunflower seeds in Tanzania are a primary source of cooking oils and animal feedstuff, mostly processed by micro-scale sunflower oil processors to yield crude oils (locally consumed unrefined by humans) and sunflower cakes locally used as animal feed. Both seeds and cakes (and perhaps solid particles in the crude oils may contain aflatoxin) are also susceptible to aflatoxin contamination. Clearly, information on the potential risk of human and animal exposure to aflatoxin via these products in this country is lacking. Also, reports on aflatoxin levels in stored sunflower seeds and cakes from micro-scale sunflower oil processors in Tanzania are

scarce. Climatic conditions, inadequate storage facilities, and suboptimal post-harvest handling of sunflower seeds by farmers and processors could be potential factors for possible aflatoxin contamination of sunflower seeds and cakes under storage conditions.

In the recent years, there have been reports that show better approaches for minimizing aflatoxin contamination in stored food crops in low-income countries. A few examples of these approaches include community-based intervention, which involved engaging the villagers to practice hand sorting of moldy nuts, proper drying of peanuts, and placing of peanut bags on wooden pallets in stores in Guinea, West Africa (Turner et al. 2005). Another approach is the use of Hermetic® triple-layer bags in Senegal (Hell et al. 2010) for limiting mold growth and aflatoxin contamination. Although these approaches have proven effective, micro-scale farmers and processors are constrained by high costs of operation (Hell and Mutegi 2011). Application of petrochemical based synthetic fungicides in stored kernels is restricted due to carcinogenic effects and non-biodegradability concerns (da Cruz Cabral et al. 2013) and there is pressure to withdraw them from the market (Pal and Gardener 2006).

Medicinal plant extracts and purified compounds that are active in inhibiting fungal deterioration of stored grains provide an opportunity to replace the synthetic fungicides. Over the years, many researchers have been devoting to searching for new antiaflatoxigenic, safe chemicals from traditional medicinal plants (Bluma et al. 2008; Bluma & Etcheverry 2008; Velazhahan et al. 2010; El-Nagerabi et al. 2013; Alejandra et al. 2013; Kedia et al. 2014; and Prakash et al. 2014). These researchers demonstrated that when *A. flavus* and *A. parasiticus* were exposed to medicinal plant extracts or pure compounds, their growth and toxin production are significantly reduced or completely inhibited. Traditional medicinal plants have been in use through their concoctions, infusions, topical solutions and root powders by indigenous people in

tropics and across the globe for decades to address their primary healthcare needs in treatment of various human ailments. The WHO admits that 80% of the global population relies on traditional medicines from medicinal plants (Bannerman 1983). In this study, we aimed to explore the potential of *Diospyros mafiensis* F. White, a medicinal shrub widely distributed in the Zanzibar-Inhambane regional mosaic in Tanzania and Mozambique (White 1988), against aflatoxin-producing molds. It is traditionally used to treat leprosy, diarrhea, and fungal skin infections (Khan et al.1980; Hamza et al. 2006).

In this study, we had two major goals. First, assessing the levels of total aflatoxin in sunflower seeds, cakes and crude oil sediments from micro-scale sunflower oil processors across Tanzania. Second, searching for safe, natural antiaflatoxigenic fungicides from *D. mafiensis* that could be used by poor farmers to protect crops in storage. Therefore, we had the following specific objectives:

1. Determine total aflatoxin concentrations in sunflower seeds, cakes, crude oil sediments from small-scale oil processors across Tanzania.
    - *Hypothesis: Levels of total aflatoxin in sunflower seeds, cakes, and crude oil sediments will exceed the regulatory limit of 20 ng/g of Food Drug Administration (FDA) and Tanzania Food and Drug Authority (TFDA).*
2. Study the anti-aflatoxigenic properties of compounds from *D. mafiensis* root bark against vegetative growth, sporulation, and aflatoxin production by *A. flavus* and *A. parasiticus*.
    - *Hypothesis: The extracts or pure compounds from the traditional medicinal plant, D. mafiensis root bark will have inhibitory effects on the vegetative growth, sporulation and aflatoxin production.*

We anticipate that the results of the first of specific objective will be useful in assessment of the risk of acquiring liver cancer in Tanzanian population exposed to total aflatoxin through consumption of sunflower seeds and crude oils and animals through sunflower cake animal feedstuffs. They will also be useful for the local food regulatory authorities to put in place aflatoxin control measures for stored sunflower seeds, cakes and crude oils from micro-scale processors in Tanzania. Post-harvest fungal deterioration and subsequent aflatoxin contamination of cereals and oilseeds during storage is a severe problem facing smallholder farmers in low-income countries leading to post-harvest losses. Antifungal studies of *D. mafiensis* might result in the discovery of affordable and alternative natural antifungals, which could be used by farmers to protect their crops under storage conditions to minimize post-harvest losses and promote food safety.

CHAPTER 1: LITERATURE REVIEW

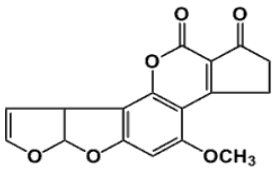
1.1 History, discovery and definition of aflatoxins

For the past five decades, humans and animals have been experiencing health threats of food and feed poisoning by natural chemical carcinogens called aflatoxins. The first threat was experienced by poultry in 1960 in England when more than 100,000 turkeys mysteriously died of unknown disease after they had been fed peanut meal imported from Brazil. Early scientists named it "turkey X disease" because the causative agents that killed that large number of turkeys were not known (Blount 1961). At about the same time other mysterious deaths of ducklings fed with peanut meals in Kenya and Uganda were reported (Sargeant et al. 1961). These incidences suggested that whatever toxic substance available in the peanut meal leading to such mysterious poultry deaths was not unique to Brazil; it was something ubiquitous. The early investigators first attributed the outbreaks to pesticides and toxic inorganic contaminants in the peanut meal, which turned to be not the case.

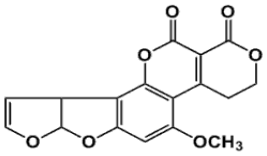
Sargeant and co-workers (1961) deserve credit for speculating that the origin of the toxin in the peanut meal could be molds, and they isolated a fungal strain called *Aspergillus flavus* from which aflatoxins were also isolated and purified. However, chemical structures of such toxins were still unknown. Professor Büch's research group at Massachusetts Institute of Technology unraveled the puzzle by elucidating their structures (Asao et al. 1963). They applied spectroscopic techniques such as infrared, nuclear magnetic resonance, ultraviolet, and mass spectrometry to define the structures of aflatoxins. Such spectroscopic interpretations revealed that the toxic substances were heterocyclic aromatic organic compounds that had a common *bis*furanocoumarin backbone fused to lactone and either cyclopentenone or cyclohexenone rings

(Figure 1.1) (Asao et al. 1963). The principal producer of these toxic food contaminants was first isolated as filamentous molds and named as *Aspergillus flavus*. The early researchers in 1960s adroitly created the name "**Aflatoxins**" for the discovered toxins by taking the letter "**A**" from *Aspergillus* and "**fla**" from *flavus* to obtain "**Afla**" that was further suffixed with "**toxins**" (Sargeant et al. 1961). Although aflatoxins were first discovered and isolated from *A. flavus*, another mold, *A. parasiticus,* became equally well known for producing aflatoxins.

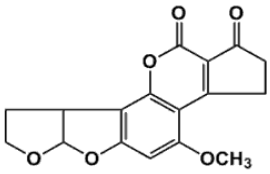
Aflatoxins (a class of mycotoxins) are defined as carcinogenic secondary metabolites (Figure 1.1) produced by molds, *A. flavus* and *A. parasiticus*. These molds colonize agricultural food and feed crops in regions where the climatic conditions (high temperature ranging from 25˚C to 35˚C, high humidity, and drought) trigger and favor mold growth and aflatoxin biosynthesis. Aflatoxin accumulation in food crops may occur both in the field and during storage, but the latter is the most critical point of severe accumulation in stored food in many countries. Aflatoxins of serious concern are of four types: aflatoxin B_1 (AFB_1), aflatoxin B_2 (AFB_2), aflatoxin G_1 (AFG_1), and aflatoxin G_2 (AFG_2) (Figure 1.1). The "B" and "G" designations are based on blue and green fluorescence observed when the pure compounds are exposed to ultraviolet light. Subscripts "1" and "2" indicate structural isomers based on their thin layer chromatographic separations (Nesbitt et al. 1962). These secondary metabolites play no significant role in the metabolism of the fungi. The fungi synthesize these metabolites as defensive chemical weapons against their predators and competitors (Ehrlich 2006).



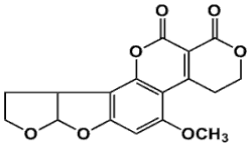
Aflatoxin B_1



Aflatoxin G_1



Aflatoxin B_2



Aflatoxin G_2

Figure 1.1: Chemical structures of aflatoxins

1.2 Aflatoxin producers, climate change and aflatoxins biosynthesis

The predominant aflatoxin producers are filamentous fungi called *A. flavus* and *A. parasiticus*. Other filamentous fungi *A. nomius*, *A. bombycis*, *A. pseudotamari*, and *A. ochraceoroseus*, have also been reported capable of producing aflatoxins; however, these are minor and rare aflatoxin-producers (reviewed in Bennett and Klich 2003). Although *A. flavus* is the most common aflatoxin-producer, the atoxigenic strains of this species have been reported to have lost their aflatoxin-producing ability due to deletions or mutations within the group of 25 continuous genes in a 70-kb cluster in the aflatoxin biosynthetic pathway (Chang et al. 2005). Defects in the regulatory gene *aflR* in the course of evolution have caused loss of aflatoxin biosynthesis capability in such strains (Matsushima et al. 2001). This loss makes them useful as biocontrol agents to exclude the aflatoxigenic *A. flavus* in the fungal communities competitively in the fields (Atehnkeng et al. 2008; Dorner et al. 2008).

The aflatoxigenic *A. flavus* strains are genetically and morphologically diverse on the basis of their aflatoxin-producing potential. Cotty (1989) delineated aflatoxigenic *A. flavus* into two main groups. The first group is the S-strain *A. flavus* that produces numerous small sclerotia

whose average diameter is less than 400 µm, and they can produce extremely high levels of B-type aflatoxins. The second group is the L-strain *A. flavus* that produces relatively few large sclerotia whose average diameter is more than 400 µm, and they produce lower levels of B-type aflatoxins. This diversity of aflatoxigenic *A. flavus* is attributed to evolutionary alterations resulting from deletions or mutations of genes within the 70-kb cluster in the aflatoxin biosynthetic pathway; thus, the 70-kb cluster length is reduced to 66.1-kb for the S-sclerotial morphotype and 66.5-kb for the L-sclerotial morphotype of *A. flavus* (Ehrlich et al. 2005).

Unlike aflatoxigenic *A. flavus*, a comparable S- and L-strain delineation of *A. parasiticus* has not yet been described. The aflatoxin biosynthetic pathways of *A. parasiticus* and *A. nomius* allow them to synthesize all four types of aflatoxins (AFB_1, AFB_2, AFG_1, and AFG_2). In contrast, aflatoxigenic *A. flavus* isolates predominantly produce only the B-type aflatoxins. Aflatoxin researchers use this distinction to diagnose the prevalence of *A. flavus* and *A. parasiticus* in their aflatoxin surveys in food and feed. For example, the presence of G-type aflatoxins in food and feed samples signals the prevalence of *A. parasiticus* although precaution must be taken not to mistake it for the unnamed SBG-strain of *A. flavus* (SBG-strain is a new and rare strain of *A. flavus* recently discovered in West Africa), which is also capable of producing the G-type aflatoxins (Cardwell and Cotty 2002; Probst et al. 2014).

Morphologically, much work has been done to distinguish the two molds without ambiguity by their characteristic colony color, conidial ornamentation, the length of conidiophore, and sterigmata arrangements, i.e., presence of biseriate or uniseriate heads (Kozakiewcz 1982; Rodrigues et al. 2007). Table 1.1 summarizes morphological differences between *A. flavus* and *A. parasiticus*. The use of Scanning Electron Microscopy (SEM) to study the morphologies of these molds has revealed noticeable characteristics on the conidial surfaces

that are diagnostically useful to separate *A. flavus* from *A. parasiticus*. For example, while *A. flavus* have conidia with smooth walls and predominantly biseriate heads, *A. parasiticus* have conidia with relatively rough walls and have predominantly uniseriate heads. Also, their colony color is used to separate the two species clearly; when grown on Czapek Dox agar (CZ) under the same conditions *A. flavus* exhibits yellow-green colonies, and *A. parasiticus* shows much darker green colonies (Kozakiewcz 1982; Rodrigues et al. 2007).

Table 1.1: Morphological differences between *A. flavus* and *A. parasiticus* (Kozakiewcz 1982; Rodrigues et al. 2007).

Characteristic	*A. flavus*	*A. parasiticus*
Colony color	Yellow-green	Ivy green (darker green)
Conidial wall ornamentation	Smooth	Rough
Seriation	Predominantly biseriate	Predominantly uniseriate
Conidiophore length	400–1000 µm	300–700 µm

The problem of aflatoxin contamination in agricultural commodities by various aflatoxin producers is inextricably linked with the issue of climate change (Wu et al. 2011). Climate influences the diversity and distribution of *A. flavus*, *A. parasiticus* and *A. nomius* species in such a way that these fungal species are more predominant in tropical climates than in temperate climates (Cotty and Jaime-Garcia 2007). It is evident that whereas aflatoxicosis outbreaks in temperate countries are rare, they are common in tropical countries because tropical climate favors aflatoxigenic fungal growth on crops (Lewis et al. 2005). Indeed, aflatoxin producers thrive best in tropical climates characterized by high humidity, high temperatures, and long periods of drought. Global warming amplifies the magnitude of such climatic conditions (Cotty and Jaime-Garcia 2007; Wu et al. 2011). Worryingly, Paterson and Lima (2010) predicted

an adverse shift of the contemporary temperate climates to new climatic conditions that will be more suitable for fungal growth. Consequently, fungal growth and aflatoxin accumulation in food and feed in temperate countries will also be as common as in tropical countries. The temperatures of temperate areas are projected to increase in the range of 30–33˚C by 2080 (National Farmers Union, 2005). In contrast, tropical climates will get incredibly warmer (> 40˚C) and might lead to either extinction of the aflatoxigenic fungi or empowerment of such fungi to produce new, harmful, secondary metabolites to cope with the new warmer climatic conditions (drought) in the tropical countries (Paterson and Lima 2010). In summary, climate change may convert "aflatoxin-free" zones to "aflatoxin-afflicted" ones by expanding the zones of aflatoxigenic fungal communities and vice versa.

Climate change alters temperatures, humidity, and rainfall patterns. It influences the distribution and severity of aflatoxigenic fungi. The spores of these fungi occur in large quantities in soils, in the air and on surfaces of crops in warmer regions than cooler areas (Shearer et al. 1992). The prevalence of the greatest aflatoxin producer, the S–strain of *A. flavus*, increases with increase in soil temperature from 16–33˚C, and the number of their spores increases as the soil temperature increases from 20–28˚C (Jaime-Garcia and Cotty 2010). These increases suggest that climate change enhances the growth of massive S–strain of *A. flavus* communities in warmer soils (Jaime-Garcia and Cotty 2010). Also, regions that receive less rain are likely to have drier soils than areas that receive more precipitation, and fungal spores available in such soils disperse more readily to the air (airborne spores) and can infect crops such as maize ears, cotton bulbs, and sunflowers (Cotty and Jaime-Garcia 2007). In the field, drought compromises crop plants such that the plants are rendered more susceptible to fungal infections. Climate shifts are directly linked to erratic and unseasonable showers. When showers occur

during or near to harvesting, they become risk factors promoting fungal growth on crops under harvest leading to unacceptable levels of aflatoxin in such crops during storage (Lewis et al. 2005; Magan et al. 2011).

Since drought causes stress to the crops, the seeds from the stressed plants become readily susceptible to insect damage, and this promotes aflatoxin contamination in seeds both in the field and in storage. As insects bore the seeds or plants, they increase the surface area for aflatoxigenic fungi to infect the seeds or plants easily. Sétamou and coworkers (1997) reported the influence of maize grain borers, *Mussidia nigrivenella*, in increasing aflatoxin levels in maize in Benin. Cotton oilseeds and sunflower oilseeds are other agricultural commodities vulnerable to insect damage (Windham et al. 1999; Hell et al. 2000; Llewellyn and Eadie 1974). Climate warming exacerbates fungal infection by increasing insect attacks in the field. Although insects are also useful for pollination, some studies have reported insects as carriers of fungal spores to the maize ear silk, cotton bulb, and sunflower heads (Klisiewicz 1979; Hell et al. 2000). This phenomenon facilitates fungal infection and aflatoxin contamination in the crops. The insect borers and aflatoxigenic fungal spore inocula in seeds may be carried over during and after harvest to the storage facilities (Magan and Hope 2003; Hell et al. 2000). Temperature elevation in the storage warehouses as a result of climate warming and humid storage conditions stimulate the metabolism of the borers to proceed aggressively with seed boring and feeding on the stored seeds throughout the storage period before processing (Jamieson et al. 2012). Concurrently, the surface area for *A. flavus* conidia inocula to grow in seeds increases and hence, aflatoxin levels also increase because the high temperatures (25–30˚C) tend to promote growth and aflatoxin biosynthesis in the granaries (OBrian et al. 2003).

Aflatoxin biosynthesis by *A. flavus* and *A. parasiticus* is a well studied and characterized physiological and biochemical process (Chanda et al. 2009; Yu et al. 2004; Roze et al. 2011). It occurs in the mold cell in response to adverse conditions such as high temperature, nutrient deprivation and oxidative stress (Chanda et al. 2009; Roze et al. 2011). It appears that aflatoxin biosynthesis plays no role in the primary metabolism and overall growth of the fungi (Bennett and Klich 2003). However, aflatoxin biosynthesis helps the producer to remove carbon in the form of acetate and nitrogen as other cofactors to protect the genome from ultraviolet radiation, to safeguard the fungi from predators (bacteria, insects, etc.), and to quench oxidative stress (Chanda et al. 2009; reviewed in Roze et al. 2011).

The starting material for aflatoxin biosynthesis is acetate, which may come from either long-chain fatty acid molecules that are converted to acetate molecules via β-oxidation of fatty acids in peroxisome or from short-chain fatty acids that are converted via the same β -oxidation to acetate molecules in the mitochondrion (Chanda et al. 2009). The synthetic scheme: acetate $\Rightarrow$ polyketide $\Rightarrow$ anthraquinones $\Rightarrow$ anthones $\Rightarrow$ aflatoxins, is an oversimplification, but it helps to highlight the starting material, a few intermediates, and the end products–aflatoxins. However, aflatoxin biosynthesis (Figure 1.2) is a complex series of conversions (from the starting substrate, acetate, to the end-product, aflatoxin), which are controlled and regulated by a 70–kb gene cluster and mediated by at least 25 enzymes leading to the formation of aflatoxins in both *A. flavus* and *A. parasiticus* (Yu et al. 2004). Yu and co-workers (2004) show highly specialized enzymes that mark the onset of the aflatoxin synthesis pathway namely alpha and beta-fatty acid synthases, and polyketide synthase (encoded in genes *fas-2, fas-1* and *pksA,* respectively). This set of enzymes orchestrates the initial conversions of acetate into the acetyl-CoA molecule, a 2-

carbon molecule condensed with two molecules of malonyl-CoA to form hexanoyl-CoA. Further condensation of hexanoyl-CoA with seven molecules of malonyl-CoA results in the production of a 20-carbon brightly red-colored compound called norsolorinic acid (NA), the first stable intermediate in the pathway (Yabe and Nakajima 2004; Yu et al. 2004; Roze et al. 2011). They occur in either peroxisomes or mitochondria of the cell depending on whether the raw materials (raw materials are usually converted to acetates by *β*-oxidation of fatty acids) are short- or long-chain fatty acids (Chanda et al. 2009). While *β*-oxidation (primary metabolism) of long-chain fatty acids occurs in peroxisomes, that of short-chain fatty acids takes place in mitochondria, but all lead to the generation of acetates (Roze et al. 2011; Chanda et al. 2009).

Production of NA triggers the activity of another set of enzymes (reductase, NA-reductase, and dehydrogenase encoded in genes *nor-1*, *norA*, and *norB*, respectively) for its conversion to an intermediate called averantin (AVN) (Yu et al. 2004). It has been shown that the genetic knockout of *nor-1*, *norA*, and *norB* in *A. flavus* and *A. parasiticus* results in NA-accumulating mutant species because this impairs the conversion of NA to AVN (Yu et al. 2004). Some other studies have indicated that this step is leaky and leads to the generation of low quantities of aflatoxins down the pathway (Detroy et al. 1973; Hong and Linz 2009). If there is no genetic blockage of such genes, aflatoxin synthesis proceeds through a series of steps until sterigmatocystin (ST), the penultimate intermediate to aflatoxins (reviewed in Yu et al. 2004).

Aflatoxins are toxic, carcinogenic chemicals that pose deleterious health effects to animals and humans. Despite their harmful effects, they do not pose such effects to the producers (aflatoxigenic fungi) themselves. The current understanding of aflatoxin synthesis from Dr. Linz's laboratory showed that there are elaborately compartmentalized infrastructures within the cell of the aflatoxin producer (Roze et al. 2011; Chanda et al. 2009) that protect the molds from

intracellular deleterious effects of the aflatoxins synthesized by the mold. These intracellular infrastructures include highly specialized vesicles, and aflatoxisomes, which are like “containers or vessels” (they are specialized organelles within the cytosol of the aflatoxin producer’s cell) in which aflatoxin synthesis is accomplished and aflatoxins safely exported and expelled to the cell exterior by exocytosis (Roze et al. 2009; Chanda et al. 2009).

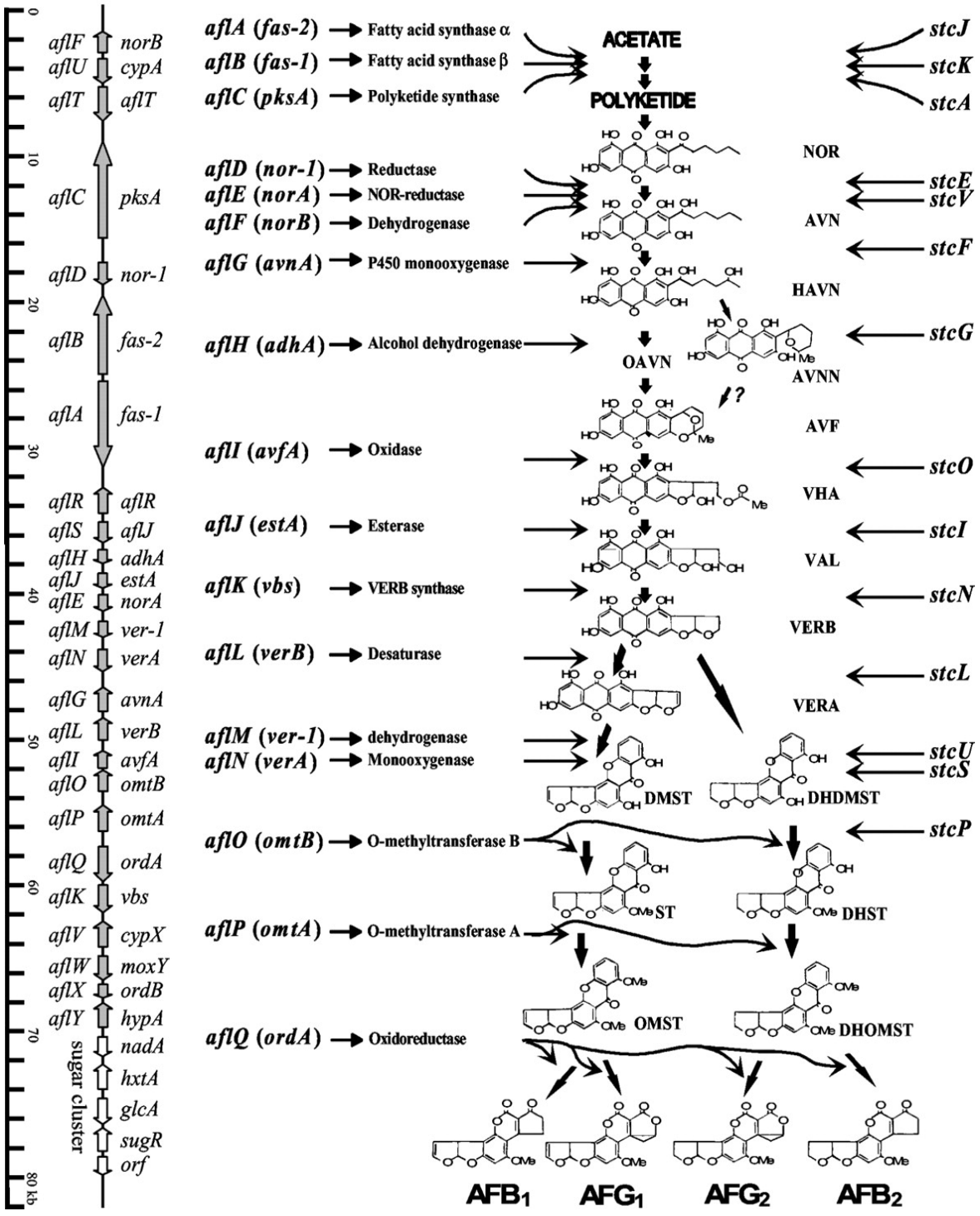


Figure 1.2: Biosynthesis pathway of aflatoxins showing a 70–kb gene cluster and intermediates leading to aflatoxins. (A) Clustered genes and (B) the aflatoxin biosynthetic pathway (Me= Methyl group) (Figure adapted from Yu et al. 2004).

1.3 Toxicity and carcinogenicity of aflatoxins

Once aflatoxin synthesis is accomplished in aflatoxisomes in the cell, aflatoxins are expelled to the extracellular space through "blast" mechanism (Roze et al. 2011; Chanda et al. 2009). When fungal growth takes place on food crops or feed, the excreted aflatoxins accumulate in food and feed. Aflatoxin synthesis by *A. flavus* results in the production of only AFB_1 and AFB_2, whereas *A. parasiticus* produces all four types: AFB_1, AFB_2, AFG_1 and AFG_2 (Figure 1.1). The International Agency for Research on Cancer (IARC, 1993) categorized AFB_1 as the most toxic and potent carcinogenic mycotoxin known. The toxicity and potency of all aflatoxins follow the order: $AFB_1 > AFG_1 > AFB_2 > AFG_2$ (Carnaghan et al. 1963). The AFB_1 is highly implicated in the occurrence of aflatoxicosis outbreaks and chronic liver cancers worldwide.

Aflatoxicosis is defined as an acute liver failure following consumption of extremely high quantities of aflatoxin. Although aflatoxicosis outbreaks in animals and humans are ubiquitous, the one that occurred in Kenya in 2004 represents the worst example ever recorded (Azziz-Baumgartner et al. 2005; Lewis et al. 2005). It killed 125 of 317 persons that were hospitalized within a week following consumption of highly contaminated maize with aflatoxins at the level of >4000 ng/g (Lewis et al. 2005). Twenty-three years earlier, Kenya had experienced previous aflatoxicosis outbreak that killed 18 of 20 patients (Ngindu et al. 1981). The patients had consumed maize contaminated with 12,000 ng/g aflatoxins. The maize was stored in damp clay granaries, which might have caused severe fungal deterioration and aflatoxin accumulation in the stored maize grains (Ngindu et al. 1981). Another deadly aflatoxicosis outbreak occurred in Western India in 1974 that caused 106 deaths of 397 hospitalized persons (Krishnamachari et al. 1975). Drought, occurrence of unseasonable precipitations during crop harvest and storage of

grains in damp storage facilities appeared to be the main risk factors for the outbreaks (Lewis et al. 2005). These conditions enable aflatoxigenic molds to accumulate extremely high levels of aflatoxins in the food and feedstuffs particularly during the storage period of damp seeds. Animals are also susceptible to aflatoxicosis. Smith and others (2007) reported an incident in which 100 dogs died of aflatoxicosis within three weeks following consumption of commercial dog feed contaminated with 598 ng/g of aflatoxin in South Carolina.

Over 4.5 billion people worldwide in the developing nations are at risk of chronic exposure to aflatoxins through contaminated food every year (Williams et al. 2004). The adverse health effects associated with chronic exposure include complications of reproductive systems (Raisuddin et al. 1993), immunosuppression (Jiang et al. 2008), growth impairment in children (Khlangwiset et al. 2011), and hepatocellular carcinoma (HCC i.e. liver cancer) (Liu and Wu 2010). The HCC is reported to be the third leading cause of cancer cases in tropical countries. Globally, WHO (2008) estimates indicate that between 25,000–155,000 persons in the tropical nations die each year of liver cancer associated with chronic exposure to aflatoxins through contaminated food (Liu and Wu 2010). Although chronic exposure to aflatoxins is a global problem, it has been mitigated in the developed countries because of technological advances which ensures routine screening of food and feeds for aflatoxins, adequate seed drying, modern storage facilities, and enforceable stringent regulatory limits. The allowable action level for aflatoxins in foods for humans in the United States, for example, is 20 ng/g. In the European Union, the allowable action level for aflatoxins is even more stringent, 4 ng/g. These regulatory guidelines have proven helpful to limit exposure to aflatoxins even though stricter limits have economic implications to the farmers (Wu et al. 2008; Wu and Guclu 2012). In contrast, tropical countries are highly vulnerable to the problem due to low technology, inadequate storage

facilities, and difficulties in enforcing regulatory laws since food and feed are homegrown at a household level. In some instances, regulatory laws may not be available (Wu et al. 2013).

The severity of the liver cancer (hepatocellular carcinoma) associated with chronic exposure to aflatoxins has been known to increase synergistically in populations with chronic hepatitis B virus (HBV) infections (Wild and Gong 2010). It is reported that populations in which HBV infections are endemic are 30 times more at risk of synergistic liver cancer cases than populations in which HBV infections are rare (Liu and Wu 2010). The HBV increases the predisposition of human hepatocytes to the carcinogenic actions of aflatoxins leading to *TP*53 mutations at codon of 249^{ser} of liver cell DNA (Kirk et al. 2005; Wild and Gong 2010). These mutations result in uncontrollable cell proliferation and malignant tumor development in the liver, which are typical features of hepatocellular carcinoma. Furthermore, the HBV antigen (HBsAg) has been studied and reported to inhibit cell DNA repair thus exacerbating liver cancer (Kirk et al. 2005).

Humans and animals get exposed to aflatoxins mainly through consumption of contaminated food and feed. Once ingested in the gastrointestinal tract, aflatoxins are absorbed and transported via hepatic portal circulation to the liver, which is the primary target organ for their hepatocarcinogenic reactions. The hepatocarcinogenic reactions are biochemical reactions mediated by cytochrome P450 (CYP450) enzymes (Gross-Steinmeyer and Eaton 2012). Aflatoxin B_1, the most hepatocarcinogenic mycotoxin, has been widely used to demonstrate the mechanism of carcinogenic actions of aflatoxins in the liver. The carbon-carbon double bond present in the furan moiety of AFB_1 is responsible for its acute toxicity and carcinogenicity. Upon arrival to the hepatocytes, the double bond undergoes an epoxidation to form two highly reactive electrophilic stereoisomers, aflatoxin B_1-8,9-endo-epoxide and aflatoxin B_1-8,9-exo-

epoxide (Figure 1.3) (Iyer et al. 1994; Wild and Turner 2002; Turner et al. 2012). The conformation of aflatoxin B_1-8,9-exo-epoxide enables it to intercalate easily between the base pairs of the DNA. Consequently, the intercalation facilitates nucleophilic reaction between the epoxide and N7 guanine to form a predominant covalently bound AFB1-DNA adduct called 8,9-dihydro-8-(N7-guanyl)-9-hydroxy-AFB_1(Kobertz et al. 1997; Wild and Turner 2002). This reaction leads to mutation of TP53 tumor suppressor gene at codon 249ser in the DNA of hepatocytes. The mutation involves transversion of G:C⇒T:A base pairs at codon 249^{ser}, which leads to the severe liver dysfunction that culminates to HCC (Kobertz et al. 1997; Kirk et al. 2005; Bedard and Massey 2006). Unfortunately, these mutations contribute to over 50% of the HCC in Sub-Saharan Africa and Eastern Asia as a result of chronic exposure to aflatoxins via contaminated food (Kobertz et al. 1997). Another toxicity aspect of AFB_1 is its high affinity for the amino acid lysine in proteins. So, it has a remarkable ability to form AFB_1-lysine albumin adducts, which are circulated in blood and they serve as biomarkers and indicators of chronic exposure to aflatoxins (Scholl et al. 1997). Biomarker data are useful for accurate estimation of dietary exposures and HCC rates in a given locality.

As a detoxifying organ, the liver uses α–glutathione S-transferase (GST) to eliminate AFB_1-8,9-exo-epoxides to prevent the formation of AFB_1-DNA and protein adducts (Figure 1.3 *vide infra*). The GST conjugates the epoxides to less toxic aflatoxin-mercapturic acids, which are excreted in urine (Scholl et al. 1997). This detoxification reaction has recently attracted the attention of chemoprevention researchers to intervene formation of AFB_1-DNA adducts (covered in depth in Section 2.5 *vide infra*). Briefly, the naturally occurring chemopreventive agents notably sulforaphane, and chlorophyllin found in cruciferous vegetables such as broccoli have been reported to boost the activity of GST to increase the rate of conjugation reaction. As a result,

the rate of generation of more mercapturic acids increases and the rate of formation of deleterious AFB_1-adducts decreases (Groopman et al. 2008; Fiala et al. 2011; Techapiesancharoenkij et al. 2015).

Another biotransformation of AFB_1 leads to the formation of milk aflatoxin (AFM_1) in which position 4 of the difuran moiety is hydroxylated (Figure 1.3). Dairy cows and nursing humans biotransform AFB_1 after they have orally ingested contaminated feedstuff and food to AFM_1 as an excretory product in milk. Thus, this transformation renders infants and milk consumers at high risk of exposure to AFM_1. Although the toxicity of AFM_1 is not fully established, there is evidence from animal models that its toxicological effects are comparable to those of AFB_1 (Cullen et al. 1987; Neal et al. 1998). Therefore, AFM_1 contamination in milk presents a huge food safety concern in the dairy industry. In the United States, for example, the maximum allowable regulatory limit for AFM_1 is 0.5 ng/g beyond which the milk has to be discarded. This contaminant in milk has necessitated FDA to set an action level of 20 ng/g for aflatoxin in animal feed because studies have shown that the dairy animals fed with feedstuffs contaminated with 20 ng/g or less of AFB_1 are likely to produce milk that is below the action level of 0.5 ng/g of AFM_1 (Price et al. 1993). Metabolites AFQ_1 and AFP_1 (Figure 1.3) are toxicologically less important, and they are beyond the scope of this review but are useful biomarkers of dietary exposure to aflatoxins.

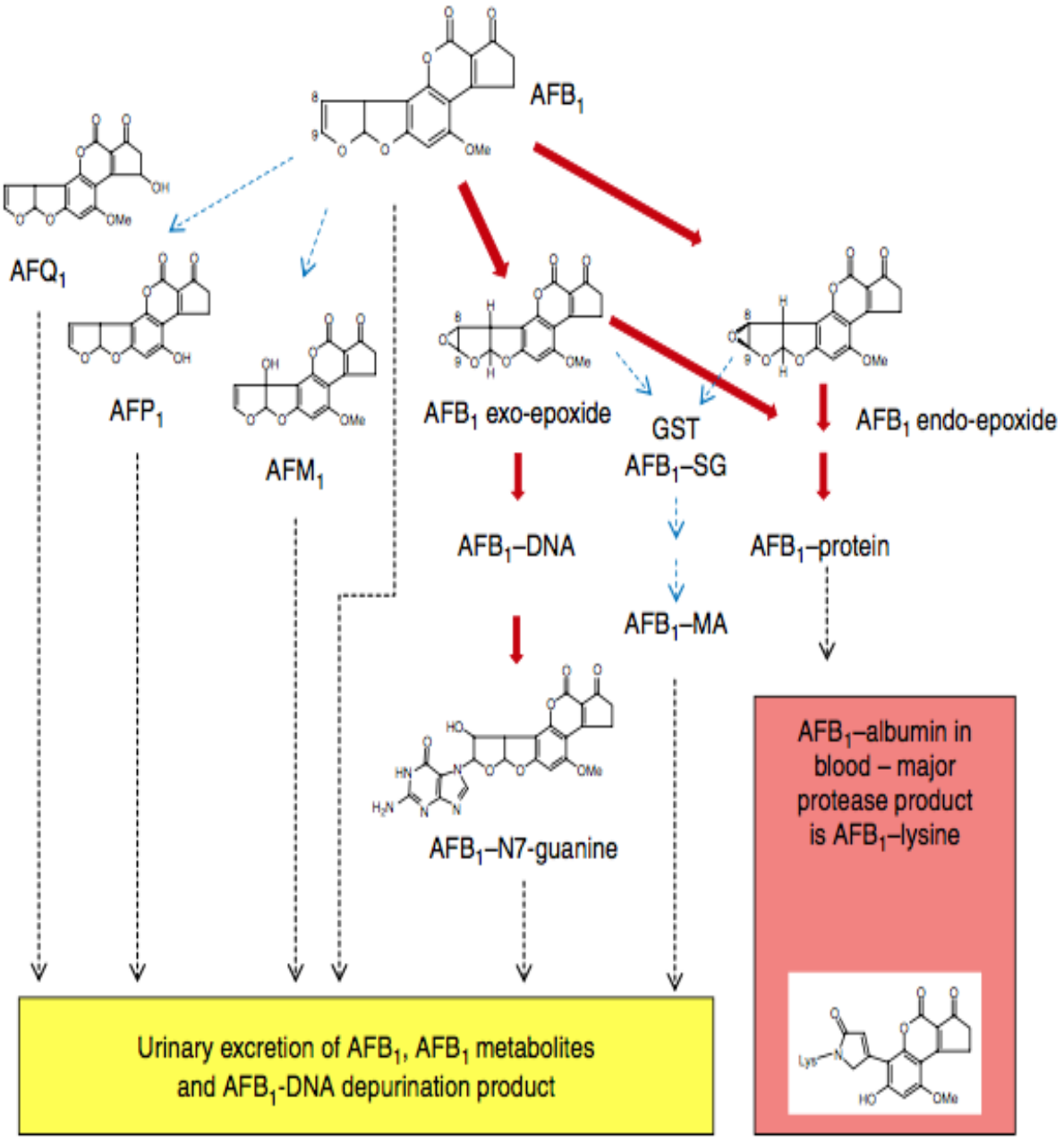


Figure 1.3: Biotransformation pathways of AFB_1 in the liver (Me = Methyl group) (Figure adapted from Wild and Turner 2002; Turner et al. 2012).

1.4 Heat stability of aflatoxins

In the food industry, heat is widely applied to cook and preserve food because it effectively kills pathogens and destroys many potential toxins in food matrices. Application of heat to destroy aflatoxins during cooking would have been the most convenient and affordable method to eliminate such toxins in food and feed in Africa and other locations where aflatoxin exposure through dietary aflatoxin is predominant. Unfortunately, aflatoxins are extremely heat-stable. Since the melting points of AFB_1, AFB_2, AFG_1, AFG_2, and AFM_1 are 267, 287, 244, 237, and 299˚C, respectively (reviewed in Milani and Maleki, 2014), it appears that thermal decomposition of these toxins requires temperatures higher than these melting points. This shows that normal cooking temperatures cannot thermally decompose the aflatoxins (Rustom 1997). Bullerman and Bianchini (2007) reported that normal cooking temperatures ranging from 100 to 120˚C do not alter the chemical structures of aflatoxins although partial thermal decomposition can occur at temperatures above 150˚. Therefore, pasteurization and sterilization of milk contaminated with AFM_1 at 100˚C cannot decompose and eliminate AFM_1 in the milk (Stoloff 1980). However, some studies contradictorily indicate that the stability of AFM_1 in milk decreases with increased storage time and cold temperatures. A study by Kiermeier and Mashaley (1977) reported a 25 % reduction of AFM_1 in milk that was stored for three days at 5˚C. The contradiction may be attributable to many factors including technical replications and the sensitivities of analytical methods in different laboratories (Rustom 1997). However, it is concluded that AFM_1 is very stable in milk such that pasteurization and sterilization cannot eliminate the toxin from the milk (Stoloff 1980).

1.5 Aflatoxin management strategies

Because of deleterious health effects of aflatoxins, in the past five decades, there has been a myriad of studies on aflatoxin control to mitigate chronic exposure of humans and animals to such toxins. Wu and Khlangwiset (2010) classified the aflatoxin management strategies into agricultural, dietary, and clinical interventions (Figure 1.4 *vide infra*). As a pre-harvest strategy, biotechnology involves identifying, genetically manipulating, and breeding agricultural crop cultivars that demonstrate resistance to aflatoxigenic fungi infestation in the field. A study by Scott and Zummo (1988) is an illustrative example of biotechnological efforts to generate maize kernel inbreds that are strongly resistant to *A. flavus*. Another study by Wu (2006) demonstrated that transgenic maize called Bt corn that was genetically engineered in America by incorporating a specific gene from the soil bacterium, *Bacillus thuringiensis*, has now found its broad adoption in America, Canada, Argentina, and South Africa due to its high resistance to aflatoxin contamination. The high resistance is due to the formation of a protein located in the maize kernel surface that is toxic to the pests including *A. flavus*. Application of biotechnology to minimize aflatoxin contamination in crops is not limited to development of highly resistant cultivars. Genetic intervention to affect the aflatoxin biosynthesis within aflatoxin producers (Section 1.2 *vide supra*) to reduce aflatoxin accumulation in food in the field has also been another strategy of interest. This strategy is based on molecular level methods to block aflatoxin biosynthesis in the aflatoxin producers to eliminate aflatoxin production (Chanda et al. 2009; Yu et al. 2004; Roze et al. 2011).

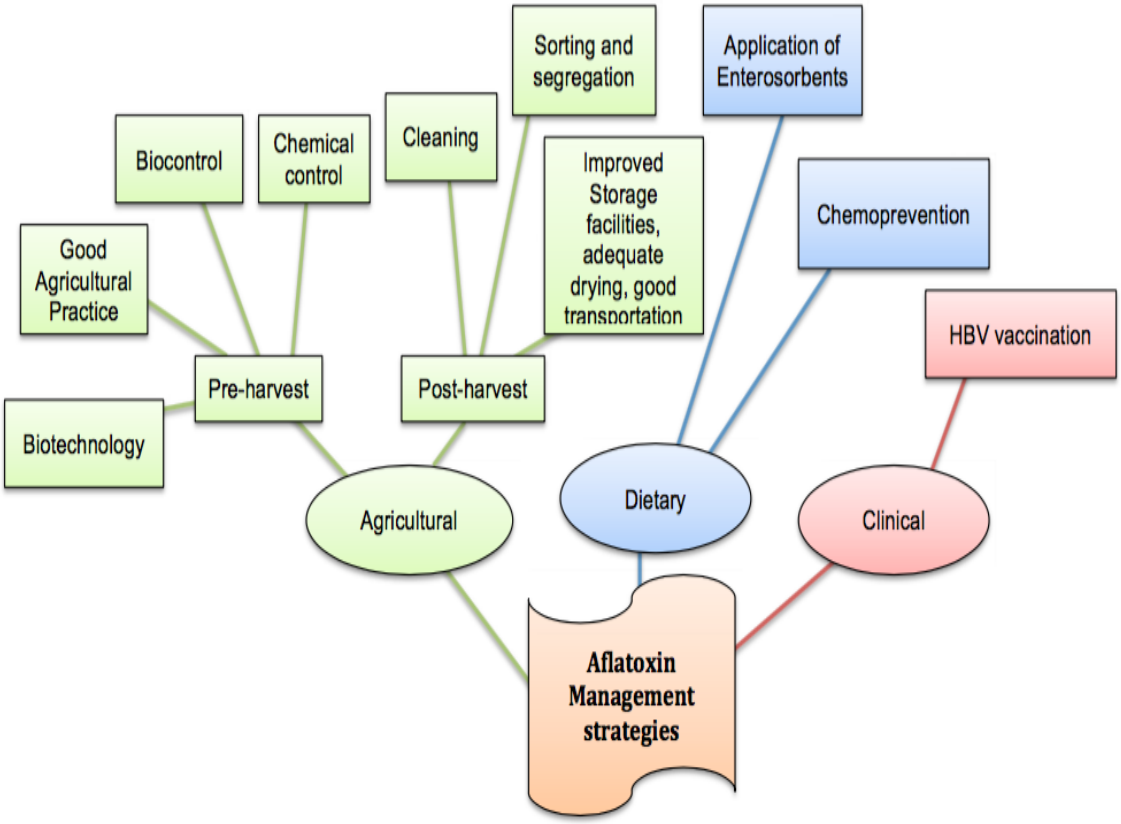


Figure 1.4: Aflatoxin management strategies for reduction of the deleterious health effects of aflatoxins (Wu and Khlangwiset 2010).

In addition to biotechnological advancement, nature has impacted and affected the genetic machinery of some of *A. flavus* strains as to cause loss of ability to synthesize aflatoxins. Such strains of *A. flavus* are non-aflatoxigenic, and they are applied in the field as biological control agents (Cotty 1994; Cotty et al. 2007; Atehnkeng et al. 2008). The United States Department of Agriculture has commercialized a biocontrol agent (Aflasafe^{TM}), which is innovatively derived from atoxigenic *A. flavus*. The maize, cotton and peanuts farmers in the Southern USA use it to exclude aflatoxigenic species in the field competitively and effectively cause aflatoxin reduction in the crops (Bandyopadhyay and Dubois 2012). Although the use of atoxigenic strains is effective to reduce aflatoxin contamination, from an ecological point of view Aflasafe^{TM} made using atoxigenic strains of one ecological zone cannot be used in fields in a

different ecological zone. This view is necessary to avoid the potential danger of destroying the indigenous fungal community structures by introducing new, exotic, atoxigenic strains (Mehl et al. 2012). The ecological consequences associated with introducing foreign atoxigenic strains for controlling aflatoxin contamination in the field are not known, but it is thought that introduction of atoxigenic strains may cause detrimental alterations of the indigenous fungal ecosystems.

One of the integrated pest management (IPM) strategies is the use of pesticides (chemical control) to manage pests such as insects to avoid insect damage of the seeds. Prevention of insect damage of seeds reduces aflatoxin contamination significantly in crops (Hell and Mutegi 2011). Insect damage of the seeds increases the surface area for *A. flavus* and *A. parasiticus* seed infestation, thus increasing aflatoxin contamination (Hell et al. 2000; Hell and Mutegi 2011). Therefore, managing insect damage of crops helps to reduce the prevalence of unacceptable levels of aflatoxin in crops. However, there are issues raised by the use of pesticides in managing insects. They include high cost, poor biodegradability, toxicity, and environmental concerns (da Cruz Cabral et al. 2013).

Aflatoxin contamination also potentially occurs in storage when post-harvest practices favor fungal growth. The practices such as storing poorly dried seed grains in poorly aerated storage facilities with poor sanitation help to promote fungal growth and subsequent aflatoxin accumulation. Aflatoxin contamination during storage is an acute problem in low-income, hot and humid countries where grains/seeds are homegrown and commonly stored in thatched clay-bamboo granaries in rural communities (reviewed in Villers 2014). Even in towns where iron-sheet-roofed stores are available, poor aeration and sanitation are the main factors that promote fungal growth and aflatoxin contamination (reviewed in Waliyar et al. 2015). Because of this

reality, Turner et al. (2005) carried out a community-based, low-tech intervention in a few villages of Guinea, West Africa that provided insight into how to reduce aflatoxin accumulation in stored grains significantly in a rural setting. Another strategy that has proven effective in reducing aflatoxin contamination during storage is the use of airtight bags called Ultra Hermetic bags^{TM} (Villers 2015). These bags limit oxygen in the stored seeds, and they prevent fungal growth and insect damage. This intervention requires that grains be adequately dried to acquire low moisture content before storage. The kernels with moisture content less than 10% can be stored for one year without fungal deterioration, provided the relative humidity is not above 70%, the temperature is within 25–27˚C range, and aeration is well maintained (Waliyar et al. 2008). Also, cleaning the storage facility and sorting the moldy kernels before storage reduces aflatoxin levels in the stored grains as compared to the unsorted grains stored in a dirty warehouse (Hell et al. 2008).

The problem of aflatoxin contamination in food and feed starts from the field (pre-harvest), and proceeds in storage (post-harvest) especially when the post-harvest handling of crops is poor. The intervention strategies known to date can only reduce the amounts of aflatoxin in food and feed, but none of them can cause complete aflatoxin elimination. Even in developed countries where the problem is less severe, low levels of aflatoxin in food and feeds are unavoidable. The FDA declared that it is impossible to have aflatoxin-free food and feed, and hence it has stringent regulations on maximum allowable action levels for total aflatoxin to protect humans and animals from deleterious effects of aflatoxins (Table 2.2).

Table 1.2 : The U.S. Food and Drug Administration action levels for total aflatoxins in food and feed (Reviewed in Rechard 2007).

Commodity	Concentration (ng/g)
Cottonseed meal as a feed ingredient	300
Corn and groundnut products for finishing beef cattle	300
Corn and groundnut products for finishing swine	200
Corn and groundnut products for breeding beef cattle, swine and mature poultry	100
Corn for immature animals and dairy cattle	20
All products, except milk, designated for humans	20
All other feedstuffs	20
Milk	0.5

Another approach to reduction of the effects of chronic exposure to aflatoxins, especially in low-income countries (Williams et al. 2004), is dietary intervention. This approach is based on the ability of chemopreventative agents to block the reaction of aflatoxin-8, 9-oxide species with hepatocytes (Egner at al. 2003). The blockage of this reaction reduces the formation of AFB_1-DNA and AFB_1-protein adducts in the liver (Egner at al. 2003). This chemoprevention intervention is defined as an application of synthetic (e.g. dithiolethiones or oltipraz) or natural anticarcinogens (e.g. chlorophyllin, sulforaphane, green tea polyphenols) to retard and/or block the hepatocarcinogenesis (Egner at al. 2003; Groopman et al. 2008; Mukhtar and Ahmad 2000; Luo et al. 2006). Chlorophyllin and sulforaphane can be extracted in cruciferous vegetables (e.g. broccoli, kale, cabbage). Green tea polyphenols (GTP) are extracted from the leaves of the tea plant, *Camellia sinesis* (Mukhtar and Ahmad 2000). The anticarcinogenic actions of these agents reduce the severity of HCC caused by aflatoxins and other environmental carcinogens in animals and humans (Groopman et al. 2008; Breinholt et al. 1995; Egner et al. 2001; Jude et al. 2007; Kensler et al. 2005; Kensler et al. 1999; Mukhtar and Ahmad 2000; Luo et al. 2006). The mode of action of these compounds is based on their ability to form complexes with aflatoxins, and most importantly on their capacity to induce the onset of the keap1-Nrf2 pathway within

hepatocytes that triggers generation of abundant liver phase II detoxifying enzymes such as glutathione S-transferase (GST)(Kwak et al. 2003). The details of the keap1-Nrf2 pathway are beyond the scope of this review. However, it suffices to mention here that GST induced by this pathway is a phase II liver enzyme whose role is to conjugate carcinogenic, reactive electrophilic species (AFB_1–8, 9–oxides) to harmless species that can readily be excreted in urine. Chemoprevention strategies using cruciferous vegetables and green tea to prevent aflatoxin-induced cancers may be practical even in low-income countries if cultivation and education on the health benefits of such crops are encouraged and promoted. However, prohibitive costs of synthetic chemopreventive agents such as oltipraz and their associated effects as a result of longtime utilization remain a considerable challenge in such countries.

Another dietary intervention strategy highly effective to prevent aflatoxins absorption in the gastrointestinal tract (GIT) is the use of commercial clay, NovaSil^{TM} (sodium calciumaluminosilicates) as enterosorbents (Philips et al. 2002). The silicates have a high affinity for aflatoxins. They readily form complexes with aflatoxins, and this property of silicates has been exploited to make clay capsules, which are used to reduce bioavailability and adverse effects of the toxins (Wang et al. 2005). The clay tablets have been experimentally tested and proven safe for animals and humans (Wang et al. 2005). Thus, the goal of this strategy is to incorporate clay capsules in food and feed or encourage people take the capsules after meals in the low-income countries where chronic exposure to aflatoxins through food is predominant (Groopman et al. 2008). This strategy has been reported to be effective in limiting bioavailability of aflatoxins to prevent severe aflatoxicosis outbreaks in humans and animals through food and feed (Wu and Khlangwiset 2010). However, this strategy is likely to suffer from negative perceptions of the humans such as dirty diets and appendicitis.

Besides aflatoxins, HBV infections are another risk factor leading to HCC. It is very unfortunate that both chronic exposure to aflatoxins through food and HBV infections are predominant in Africa and Asia (Wild and Gong 2010). The interactions of the deleterious effects of aflatoxins and HBV in the hepatocytes are synergistic in nature. They are 30–fold more severe than the sum of their independent effects (Wild and Gong 2010; Liu and Wu 2010). Mechanistically, HBV tends to predispose the DNA of hepatocytes to aflatoxins thereby facilitating intercalation between DNA and the toxins. Between 60–80% of HCC deaths worldwide are attributed to the synergetic severity of chronic exposure to aflatoxins and HBV infections of the liver (reviewed in Moudgil et al. 2013). Morbidity and mortality associated with HCC in populations where HBV infections are prevalent are reduced by antiviral treatments using HBV vaccines. According to WHO 2000, such vaccines have been in use since 1982 in many low-income countries, and they are effective in decreasing the incidence of HBV infections among infants, children, and adolescents (Lavanchy 2005). Vaccination, in turn, has helped to minimize the synergistic effects of both HBV and aflatoxins in low-income countries.

The complexity of the problem of aflatoxin contamination in food and feed demonstrates there is no singularly successful aflatoxin management strategy. The combination of as many strategies as possible can yield a synergistic, positive impact to protect humans and animals from chronic exposure to aflatoxins and HCC.

1.6 Aflatoxin research in Tanzania

Countries or parts of countries located within latitudes 40˚N and 40˚S worldwide are more vulnerable to chronic exposure to aflatoxins than those outside this range of latitudes because of climatic conditions favorable to *A. flavus* and *A. parasiticus* infection of crops (Williams et al. 2004). Tanzania is an East African country located at latitude 6˚S away from the equator, and it is within this region of chronic exposure to aflatoxins.

Our literature survey on aflatoxin research in Tanzania indicates occurrence of aflatoxin in crops such as beans (Seenappa et al. 1981), cowpeas (Seenappa et al. 1983), cured fish (Mugula and Lyimo 1992), cassava (Manjula et al. 2009), and maize (Kimanya et al. 2008; Kamala et al. 2015). Studies on dietary co-exposure of Tanzanian children to aflatoxins and fumonisins through complementary foods have been reported (Shirima et al. 2013; Kimanya et al. 2014; Magoha et al. 2014). These studies collectively demonstrate that Tanzanian population is chronically exposed to mycotoxins including aflatoxins through foods such as beans, cowpea, maize and cassava. However, little attention has been paid on exposure to aflatoxins through crops such as sunflower. This crop is used as a primary source of cooking oils, powders as soup thickeners, snacks, and cakes as animal feed. Farmers use sunflower cakes as animal feed for dairy cattle, yet the prevalence of aflatoxin in those feedstuffs has never been known. The problem associated with the use of aflatoxin-contaminated feedstuffs is the presence of AFM_1 in cow milk retailed to the public as reported recently in Dar es Salaam, (Urio et al. 2006), and in Singida (Mohammed et al. 2016) in Tanzania. Nursing mothers chronically exposed to AFB_1 via various kinds of contaminated foods including sunflower products are likely to bear AFM_1 in their breast milk as reported recently in Northern Tanzania (Magoha et al. 2014). Thus, our first objective of this dissertation was to survey and determine the levels of total aflatoxin in

sunflower seeds, crude sunflower oils, and cakes locally produced in central sunflower corridor (CSC) in Tanzania.

1.7 Susceptibility of sunflower to aspergillus fungal attack

There is evidence that like maize, cassava, and peanuts, oilseed crops such as sunflower, sesame, and cotton are also susceptible to aflatoxigenic fungal infection in that field and storage facilities (Chakrabarti 1987; Jamie-Carcia and Cotty 2004; Banu and Muthumary 2005; Nyandieka et al. 2014). The primary reason for their susceptibility is that these seeds are good sources of fatty acids, which are the raw materials required by the molds for aflatoxin biosynthesis (Chakrabarti 1987). According to Chakrabarti (1987), molds can increase the ratio of saturated to unsaturated fatty acids so as to increase the quantity of saturated fatty acids. Saturated fatty acids stimulate aflatoxin biosynthesis probably by favoring the biochemical conversion of saturated fatty acids to acetates and polyketides in the cytosol of the mold cell (Section 1.2 *vide supra*). Thus, sunflower oilseeds are susceptible to fungal infection and contaminated seeds results in aflatoxin contamination in the resultant sunflower cakes and crude oils after milling. Metabolic conversion of unsaturated fatty acids to saturated ones increases the oil content of undesirable trans fatty acids in the final product. Furthermore, the final oils produced from moldy oilseeds tend to be thicker and more viscous as compared to the oils produced from the sound non-deteriorated oilseeds (Chakrabarti 1987).

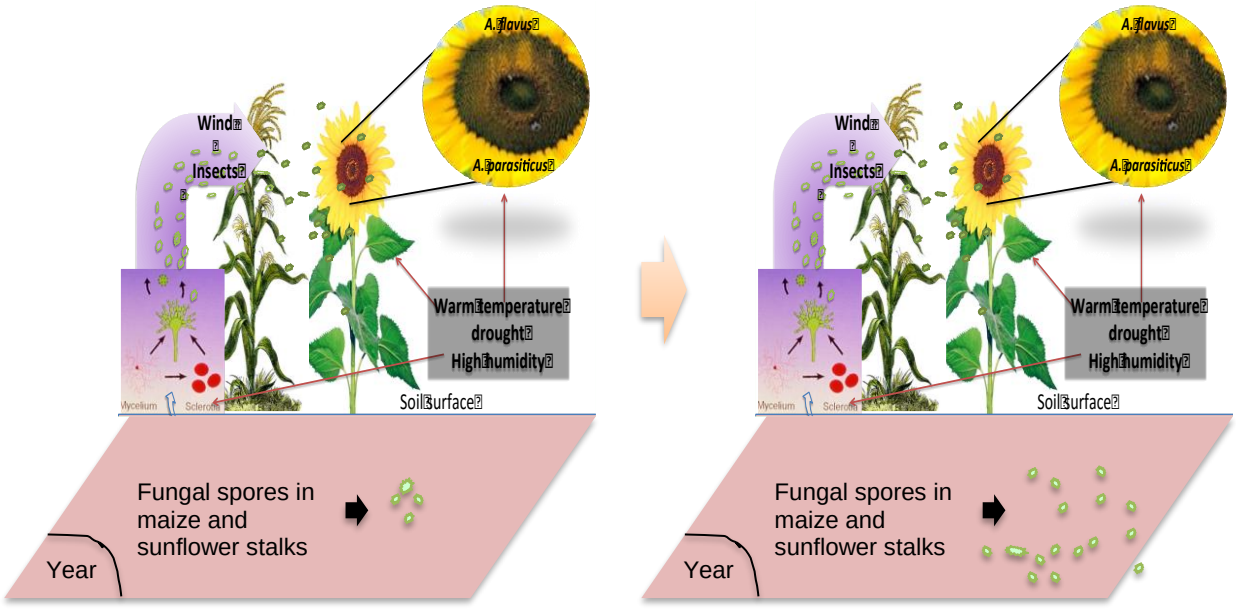


Figure 1.5: Soil enrichment of aflatoxigenic spores. Wind and insects are the primary dispersing agents of aflatoxigenic spores from the soil and stalks to the surfaces of crop plants. Maize is very susceptible to fungal infection. Intercropping sunflower with maize increases the likelihood of cross contamination from maize to sunflower. Also, recycling and intercropping of crops highly susceptible to fungal attack will continue to grow conidia and spore densities in the soil from year 1 to year 2, etc. as long as their favorable conditions (drought, humidity, and warmer temperatures) continue to persist (Figure modified from Jouany 2007).

In Tanzania, micro-scale sunflower farmers often intercrop maize with sunflower in the same field (Figure 1.5). Maize is the most vulnerable crop to the fungal invasion, particularly when it is confronted by adverse weather conditions (drought, humidity, and warmer temperatures). Jaime-Garcia and Cotty 2004 reported the ability of maize cobs and stubbles to harbor aflatoxigenic conidia that serve as potential sources of *A. flavus* and *A. parasiticus* spores (inocula) to propagate inoculation in the subsequent farming seasons. These researchers noticed that maize cobs and stubbles in the maize plantations in South Texas, USA harbored over 190 times more *A. flavus* propagules than the soil without such debris materials. Thus, cycling and intercropping of maize with other susceptible crops may increase aflatoxigenic mold densities in

the fields in the subsequent years. Unfortunately, insects and the wind disperse the spores from the soil and stubbles to the surfaces of crop plants in the field (Klisiewicz 1979; Hell et al. 2000). The traditional practice of micro-scale farmers of burning maizecobs and sunflower stubbles before the new farming season is reported to be useful to reduce aflatoxigenic spores densities in the soil (Jouany 2007). However, this practice is non-selective–it eliminates even the innocent but ecologically important soil-borne flora in the field. Therefore, harvested sunflower seeds from the field are likely to contain aflatoxigenic spores that are carried over to transportation facilities, and deterioration continues to and during storage.

1.8 Potential of traditional medicinal plants for aflatoxin reduction in storage

The World Health Organization (WHO) has estimated that over 80% of the people in the low-income countries depend on traditional medicines to meet their primary healthcare needs (Bannerman 1983). Most of these are poor people who cannot afford the pharmaceutical drugs and do not have medical health insurance. Culture and economics are the primary reasons for extensive use of traditional medicines over the modern pharmaceutics in many developing countries in which the majority of the users are the rural populations who have little or no access to health workers, and dispensaries (Sawadogo et al. 2012). Traditional healers and practitioners are their options from whom they obtain the traditional healthcare more affordable. Due to insufficient technology and financial resources, traditional healers locally make concoctions, infusions, decoctions, poultices and powders added to porridge as recipes for treating diseases (Holmstedt and Bruhn 1983; De Boer at el. 2004; Sawadogo et al. 2012). Only the elite and the wealthy (usually live in urban areas) can afford medical health insurances and pharmaceutical drugs (Sawadogo et al. 2012). Thus, the importance of traditional medicines from medicinal plants to the poor people for their primary healthcare worldwide cannot be overemphasized. That

is why during the 1978 Alma Ata conference, the WHO officially recognized the contribution of traditional medicines to promote the public health. It encouraged continuous scientific investigation of medicinal plants and integration of the traditional medicines into national health programs as a more plausible means of reaching the global goal of primary healthcare for all (Awodele et al. 2011). Tanzania as one of the WHO member countries endorsed the call to recognize traditional medicine and practitioners by passing the Traditional and Alternative Medicine Act in 2002. This Act recognizes and integrates traditional medicines in the national health system and bridges the gap between the traditional healers and regular physicians (Stangeland et al. 2008).

What has traditional medicines from plants to do with the management of aflatoxin in crops? The flora of Tanzania is endowed with over 10,000 medicinal plant species (Nahashon 2013). Many of these herbs have been reported to have antiviral, cytotoxic, anticancer, antitumor, antimalarial, antibacterial and antifungal properties. Thus, they have been used to treat ailments such as skin rashes, cancer, candidiasis, malaria, headache, diarrhea, toothache, and fungal diseases (Hamza et al. 2006; Runyoro et al. 2006; Van Den Bout-van Den Beukel et al. 2008; Sawadogo et al. 2012; Musila et al. 2013). However, there are limited reports on potential antiaflatoxigenic agents from medicinal plants that could be used to knock deleterious molds out of the crops in storage systems. Medicinal plants have the inherent ability to produce secondary metabolites such as alkaloids, steroids, flavonoids, isoflavonoids, tannins, cumarins, glycosides, termpens, phenylpropannes, terpens, phenylpropannes, and organic acids (da Cruz Cabral et al. 2013). These metabolites protect the plants against their predators, competitors, pests, pathogens and environmental stress (reviewed in Roze et al. 2011 and da Cruz Cabral et al. 2013).

Apart from the provision of primary healthcare for poor people, can medicinal plants provide protection against agricultural losses and diseases caused by fungal deterioration of food crops? This dissertation was interested in the impact of traditional medicinal plants on food safety and thus, public health (Figure 1.6). The effects are predicted to be synergistic (?) on animal and public health when these three components are interwoven.

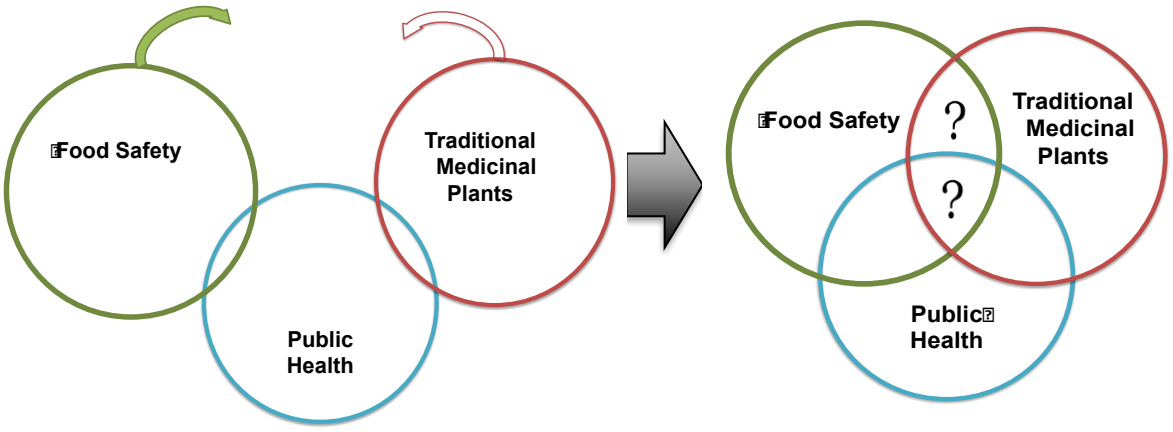


Figure 1.6: The closure of food safety and traditional medicinal plants bridge. The importance of safe food to public health as well as the importance of traditional medicinal plants to public health has well been characterized. What traditional medicinal plants can do to promote food safety is explored in this study.

Because medicinal plants are rich in a wide of variety of bioactive compounds, it is thought that they may offer better, affordable, and biodegradable alternative fungicides to protect crops from fungal deterioration during storage. *A. flavus* and *A. parasiticus* deteriorate a broad range of food crops and produce aflatoxins (liver cancer-causing agents) that accumulate in such crops. Researchers in this area have reported that essential oils, extracts, powders and pure compounds derived from many medicinal plants can inhibit growth and production of aflatoxins by *A. flavus* and *A. parasiticus* (El-Nagerabi et al. 2013; Alejandra et al. 2013; Bluma et al. 2008; Bluma and Etcheverry 2008; Kedia et al. 2014; Prakash et al. 2014 and Velazhahan et al. 2010).

The oil extracted from *Chenopodium ambrosioides* inhibited completely AFB_1 production by *A. flavus* at a concentration as low as 10 µg/mL (Kumar et al. 2007). In another study, Reddy and coworkers (2009) discovered a complete growth inhibition of *A. flavus* and subsequent toxin production using *Syzigium aromaticum* oil extracts at 5000 µg/mL. A medicinal plant, *Piper betle* L., produces the essential oil that is strongly inhibitory to aflatoxigenic producers even at a very low concentration of 0.6 µg/mL although at 0.1 µg/mL there was a very low inhibition accompanied by higher aflatoxin production than the control (Prakash et al. 2010). These authors concluded that molds exposed to low doses of the plant extract could be so aggressive as to produce more aflatoxins than normal to defend themselves against the adverse stress brought to their immediate environment but they get strongly inhibited at high doses.

Besides essential oils, many medicinal plants are rich in phytochemicals containing phenolic ring structures that promote delocalization of π-electrons within the rings and attached functional groups (e.g. epoxides, hydroxyl, amides, etc.) that have been reported to be strongly anti-mold (Souza et al. 2005; da Cruz Cabral et al. 2013). These are polar compounds; they are efficiently extracted from their plant matrix using polar solvents such as methanol, ethylacetate, and acetone because polar substances dissolve well in polar solvents. Also, these solvents permeate the plant material well to afford efficient extraction of polar compounds (Sultana et al. 2009).

Although the mode of action of these phytochemicals is not clearly understood, it is theorized that their inhibitory effects on molds are associated with interactions of the functional groups (epoxides, hydroxyl, amides, aromatic rings, etc.) of the phytochemicals with the cellular components of the molds (da Cruz Cabral et al. 2013). For example, the hydroxyl and epoxide groups of the phytochemicals are capable of forming hydrogen bonds with proteins, and DNA,

thus, altering the integrity of the mold cells. Exposure to phytochemicals interferes with the metabolic processes such as respiration and aflatoxin biosynthesis. Phytochemicals are biodegradable and environmentally user-friendly. Additionally, compounds from traditional medicinal plants may have less toxic effects to humans and animals as such plants have been used since medieval times in form of concoctions, infusions, decoctions, poultices and powders to treat human diseases (Holmstedt and Bruhn 1983; De Boer at el. 2004; Sawadogo et al. 2012). The fact that plant extracts contain a myriad of compounds of mixed functional groups could also offset the issue of mold resistance to the conventional pesticides (e.g. thiabendazole, imazalil, and sodium *ortho*-phenylphenate). Aside from resistance to molds, the indiscriminate use of conventional pesticides has also raised public concerns about risks from exposure to synthetic fungicides residues in food (da Cruz Cabral et al. 2013). The second objective of this dissertation was to study the potential of *Diospyros mafiensis* F. White from Tanzania against *A. flavus* and *A. parasiticus* growth and toxin production that could be used as safe post-harvest crop protectants.

CHAPTER 2: AFLATOXIN LEVELS IN SUNFLOWER SEEDS, CAKES, AND CRUDE OIL SEGMENTS COLLECTED FROM MICRO- AND SMALL-SCALE SUNFLOWER OIL PROCESSORS IN TANZANIA

Portions of this chapter are included in a manuscript which has been submitted to PLoS ONE for publication: **Mmongoyo JA, Linz JE, Wu F, Nair MG, Mugula JK, Strasburg GM**

2.1 Abstract

Aflatoxin, a mycotoxin found commonly in maize and peanuts worldwide, is associated with liver cancer, aflatoxicosis and growth impairment in humans and animals. In Tanzania, sunflower seeds are a source of snacks, cooking oil, and animal feed. These seeds are a potential source of aflatoxin contamination. However, reports on aflatoxin contamination in sunflower seeds and cakes are scarce. Our objective was to determine total aflatoxin concentrations in sunflower seeds, cakes and crude oil sediments from small-scale oil processors across Tanzania. Sunflower seed samples (n = 90), cake samples (n = 92) and crude sunflower oil sediments (n = 50) were collected across two years and analyzed for total aflatoxin concentrations using a direct competitive enzyme-linked immunosorbent assay (ELISA).

For samples collected from June-August 2014, the highest aflatoxin levels in sunflower seeds were from the towns of Babati-Manyara (162.0 ng/g), Singida (261.8 ng/g) and Dodoma (280.6 ng/g). The concentration ranges were 1.8–162.0, 1.4–261.8, and 1.7–280.6 ng/g, respectively. For the cake samples the highest aflatoxin levels were from the towns of Singida (34.3), Dodoma (88.2), and Mbeya (97.7 ng/g). The concentration ranges were 2.0–34.3, 1.9–88.0, and 2.8–97.7 ng/g, respectively.

For samples collected August-October 2015, the highest aflatoxin concentrations in sunflower seeds were from the towns of Mbeya (174.2 ng/g), Singida (217.6 ng/g), and Morogoro (662.7 ng/g). The concentration ranges were 1.4–174.2, 1.6–217.6, and 2.8–662.7 ng/g, respectively. For the cake samples, the highest aflatoxin concentrations were from the

towns of Singida (52.8 ng/g), Morogoro (536.0 ng/g), and Dodoma (598.4 ng/g). The concentration ranges were 3.2–52.8, 2.7–536.0, and 1.4–598.4 ng/g, respectively.

For crude sunflower oil sediments collected June-August 2014, total aflatoxin could not be detected in the majority samples. However, it was detected in one sample of each of the following: Singida (6.5 ng/g), Dodoma (3.8 ng/g) and Morogoro (5.0 ng/g). For crude sunflower oil sediments collected August-October 2015, there was no contamination in oil sediments of Manyara, Singida, and Dodoma, but nearly all samples from Morogoro were found contaminated (2.4, 85.3, 8.7, 41.7, and 12.9 ng/g).

We estimated human aflatoxin exposure through sunflower consumption to be 25 and 21 ng kg/bw/day and the associated population risk for liver cancer case values of 0.91 and 0.77 cases per 100,000 for Dodoma and Manyara, respectively based on the 2014 samples. Based on the 2015 samples, aflatoxin exposure was 23.8 ng/ kg/bw/day and population risk for liver cancer case value of 0.86 cancer cases per year per 100,000 for Morogoro. Human aflatoxin exposure through crude oil extracted from sunflower seeds was considerably lower. We concluded that Tanzanians were potentially at risk of exposure to aflatoxins through sunflower seeds from micro-scale oil millers Dodoma, Morogoro, and Manyara in Tanzania. It appears that the geographic source of the sunflower seeds in Tanzania influences risk of aflatoxin exposure.

2.2 Introduction

Aflatoxins are secondary metabolites produced by the fungi *Aspergillus flavus* and *Aspergillus parasiticus*, which commonly infect food crops such as maize, peanuts, and tree nuts. They cause liver cancer and aflatoxicosis in humans and animals. The fungi produce four main types of aflatoxin: aflatoxin B_1 [AFB_1], B_2 [AFB_2], G_1 [AFG_1], and G_2 [AFG_2]. AFB_1, the most carcinogenic mycotoxin, is typically produced in higher quantities than its counterparts. The International Agency for Research on Cancer (IARC) has classified "naturally occurring mixes of aflatoxins" as a Group 1 human carcinogen: known to cause cancer in humans (IARC 1993).

Chronic exposure to aflatoxin contributes to increased incidence of liver cancer cases worldwide. It has been estimated that 25,000–155,000 humans die each year of liver cancer associated with chronic exposure to aflatoxins, through consumption of contaminated maize and peanuts (Liu and Wu 2010). Furthermore, chronic exposure to dietary aflatoxin is associated with immunosuppression (Jiang et al. 2008), stunted growth in children (Khlangwiset et al. 2011), and acute aflatoxicosis at high doses (Strosnider et al. 2006). In the past, human and animal exposure to dietary aflatoxins in Sub-Saharan Africa was considered to be mainly through consumption of maize and peanuts. However, consumption of oilseeds such as sunflower, sesame, and cotton may also contribute significantly to the overall human and animal exposure to aflatoxins through food and feed (Elzupir et al. 2010; Idris et al. 2010; Kang'ethe and Lang'a 2009).

In Tanzania, sunflower is an oilseed crop that primarily provides animal feed and cooking oil, as well as snacks for humans. The Central Sunflower Corridor (CSC), comprised of Mbeya, Iringa, Morogoro, Dodoma, Singida, Manyara and Karatu-Arusha, leads in sunflower farming and sunflower micro-scale oil milling activities. A report by the Tanzanian Ministry of

Agriculture, Food Security and Cooperative (MAFSC) indicates that national annual production in 2008 was approximately 350,000 metric tons (RLDC 2008). In 2015, production had increased about tenfold from 2008, driven primarily by an increased sunflower seed market. Therefore, the sunflower industry is an important contribution to the economics of Tanzania.

Small-scale sunflower farmers make a living by selling sunflower seeds to processors, who extract cooking oil and produce seed cakes. In 2015, a 70 kg–bag of sunflower seeds was sold for 60,000 Tanzania Shilling (Tshs) (US$30), and the processing could produce approximately 45 kg of cakes and 20 liters of crude oil for sale. A 5 kg-loss might be due to the poor efficiency of the milling machines. While humans eat roasted and salted seeds as a snack food, the cakes are used as animal feed for chickens, dairy cows, and goats. Dodoma, Singida, Arusha and Manyara are the major sunflower cake-producing regions in Tanzania; producing approximately 100,000 metric tons of sunflower cakes per year, which serve as a reliable source of animal feed for livestock in Northern Tanzania and Kenya.

The surveillance of aflatoxin levels in sunflower cakes is critical because the cakes are an important constituent of dairy cattle feed, and dairy cattle can transform AFB_1 to AFM_1 and excrete the latter in milk. While AFM_1 is much less carcinogenic than its parent compound (Cullen et al. 1987), the health effects are still not fully established and many nations have set regulatory standards to control its presence in milk. Additionally, aflatoxin in animal feeds causes illness in animal husbandry and loss in the meat industry.

Total aflatoxin concentrations in sunflower seeds and cakes produced in Tanzania have not been analyzed or reported, leaving questions such as to what magnitude of the risk to human and animal health from consumption of sunflower products. Therefore, the aim of the present study was to survey total aflatoxin levels in sunflower seed and cake samples collected from multiple

micro-scale sunflower oil mills across Tanzania, and use mean contamination levels to estimate exposure and characterize the risk of primary liver cancer in the Tanzanian population.

2.3 Materials and Methods

2.3.1 Collection of samples

In the 2014 harvest season (June–July), a total of 128 samples of sunflower seeds (S), cakes (C), and crude sunflower oils (O) samples were collected from sunflower processing facilities across Tanzania. The sunflower seed and cake samples were each about 200 g and sunflower crude oil samples were each about 50 mL. The seed samples (S = 42), seed cake samples (C = 44) and crude sunflower oils (O = 42) were randomly collected from individual sunflower oil extractors in the following towns: Mbeya (S = 7; C = 7; O = 7), Iringa (S = 7; C = 7; O = 7), Morogoro (S = 5; C = 5; O = 4), Dodoma (S =7; C = 7; O = 7), Singida (S= 6; C = 6; O = 6), Babati-Manyara (S = 6; C = 7; O = 6), and Karatu-Arusha (S= 4; C = 5, O = 5).

In 2015 (September–October), a total of 144 samples were collected. The seed samples (S = 48), seed cake samples (C = 48) and crude sunflower oils (O = 48) were randomly collected from sunflower oil extractors in the following towns: Mbeya (S= 9; C = 9; O = 9), Iringa (S = 7; C = 7; O=7), Morogoro (S = 6; C = 6; O = 6), Dodoma (S = 7; C = 7; O = 7), Singida (S = 7; C = 7; O = 7), Babati-Manyara (S = 7; C = 7; O = 7) and Karatu-Arusha (S = 5; C = 5; O = 5).

All samples were placed in polyethylene bags and taken to Sokoine University of Agriculture for aflatoxin analysis. All samples were stored at -20 ˚C prior to analysis.

2.3.2 Materials and chemicals

Veratox for aflatoxin ELISA kit (8030, Neogen Corporation, Glasgow, UK) consisted of aflatoxin standards 0, 5, 15, 50 ng/g; antibody wells, conjugate, substrate, and stop reagent;

Veratox® Mycotoxin Starter Kit (9271A); Mycotoxin Extraction Kit (8052); and Neogen 4700 Micro-well Reader (9303). These materials were purchased from NeogenEurope Corporation (Reg. No. 18634, St Stephen's House, 279, Bath Street, Glasgow, G2, 4JL, UK). HPLC–grade methanol (Sigma–Aldrich, St. Louis, MO, USA) was used as received. Stock AFB_1 standard (10 µg in 10 ml methanol) was purchased from (Trilogy Analytical Laboratory Inc. Washington, MO, USA). Deionized water was obtained from Sokoine University of Agriculture, Morogoro, Tanzania.

2.3.3 Aflatoxin analysis

2.3.3 (a) Extraction of total aflatoxin from sunflower seed meals and cakes

Aflatoxins from the seed and cakes were extracted using an AOAC-approved method (AOAC-RI 050901) as recommended by Neogen Corporation. A representative sample (~200 g) of seeds or cakes was thoroughly ground into fine powder using a mill grinder (IKA® A11 Basic 07.028450, IKA® Works, Inc., 2635 North Chase, NC 28405–7419, Wilmington, USA). Then, 50 mL of methanol/deionized water (70:30 v/v) were added to the powdered sample (10 g) in a mycotoxin extraction cup (250 mL) to make a suspension, which was vigorously shaken for 3 min. The suspension was allowed to rest until all particles settled to the bottom. The supernatant solution was then decanted, and filtered into a sample tube using a syringe filled with the cotton wool filter. The pH values of all sample solutions ranged from 6.7 to 7.4.

2.3.3 (b) Extraction of total aflatoxin from crude oil sediments

The crude oil sample placed in a 15-mL or 50-mL falcon tube was centrifuged using (Allegra®X-15R Centrifuge, Beckman Coulter Inc., 4300N, Fullerton California 928 34-3100, USA) at 1000 rpm (relative centrifugal force = 205) for 2 h at 10˚C to obtain solid particles (sediments). Using plastic disposable pipets, clarified oil was separated from the sediments deposited at the bottom

of each tube. Weights of the sediments are shown in Appendix 1 *vide infra*. One gram of each sediment was placed in a separate tube and to this tube, 5 ml of 70 % methanol was added. The suspension was vortexed for 3 min and then centrifuged for 1 h to obtain the aqueous methanolic extract. The methanolic extract was collected and transferred to a fresh tube. The pH values of the sample extracts ranged from 6.08 to 7.81.

2.3.3 (c) Enzyme-linked immunosorbent assay

The extracts were tested for aflatoxins using Veratox Direct Competitive Enzyme-Linked Immunosorbent Assay (ELISA) in a micro-well format as indicated by the manufacturer (Neogen Europe Corporations, Glasgow, UK) and Manjula et al. (2009). The limit of detection (LOD) of this assay was 1.4 ng/g. The concentration below the LOD was reported as not detected (n.d.). The concentration that exceeded 50 ng/g (the highest concentration of standard) was further diluted. The diluted sample concentration was multiplied by the dilution factor to obtain the actual concentration of total aflatoxin in the original sample. All samples were analyzed in triplicate to obtain mean contamination concentrations.

2.3.3 (d) Recovery of AFB_1

The sensitivity of the method was determined by determining percent recovery of aflatoxin for the seeds and cakes. Using a Hamilton-Syringe–fixed needle (Lot 719446; Hamilton Company, Reno, Nevada), aflatoxin-free sunflower seeds and cakes powders (among the samples) (10 g) were spiked with AFB_1 standard at 10 and 25 ng/g concentrations (Table 2.1). AFB_1 spiked samples were extracted according to AOAC-RI 050901 method to recover AFB_1. The spiked samples were analyzed in triplicate for each spiked concentration, and averages and standard deviations were determined.

2.3.4 Exposure assessment and risk characterization for humans

Exposure to aflatoxin across a particular population can be estimated by multiplying median contamination levels of aflatoxin in the food consumed and the daily intake rate of the food, and dividing by body weight. Thus, exposure estimates were calculated using daily intake rates data of sunflower seeds, and oils relevant to various regions of Tanzania. The daily intake rates data were obtained from Tanzania National Panel survey 2011 (TNPS 2011); http://catalog.ihsn.org/index.php/catalog/4617. The FDA average bodyweight of 70 kg was used to calculate exposure estimates to total aflatoxin using the following equation:

Exposure = (Median Contamination Level x Daily Intake Rate)/ (Body Weight of a Consumer) (Equation 1).

Apart from chronic dietary exposure to aflatoxin, hepatitis B virus (HBV) is also a risk factor for liver cancer in low-income countries (Groopman et al. 2005). In Tanzania chronic HBV infection is about 9% (Wu and Liu 2010). To characterize the population risk of aflatoxin-induced liver cancer in this population, we assumed that 9% of the population is HBV-positive and 91% is HBV-negative to derive a combined aflatoxin-induced liver cancer potency factor for the Tanzanian population. The Joint FAO/WHO Expert Committee on Food Additives (JECFA) derived potency factor of 0.01 cases per 100,000 per year per ng/kg bw/day aflatoxin exposure for populations without chronic HBV infection, and 0.30 for populations with chronic HBV infection (JECFA 1998). Therefore, an average (combined) liver cancer potency factor relevant to Tanzania was obtained by using the following equation (Shephard 2008):

Average (combined) potency factor = (0.01 x 0.91) + (0.3 x 0.09)

= 0.0361 cases per year per 100,000 per *n*gAFB$_1$ kg/bw/day

(Equation 2).

We used this average potency factor to estimate population risk of acquiring liver cancer as a result of known daily intake per day of seeds and crude oils, and average aflatoxin levels in the sunflower products in Tanzania. Our data provided us with inputs for the variables in Equation 1.

Population risk = Exposure estimate x Average potency factor (Equation 3).

2.4 Results

2.4.1 Recovery of AFB_1

Recovery of AFB_1 in the test sample was greater than 70% (Table 2.1), indicating the consistent performance of the approved AFB_1 extraction protocol (AOAC-RI 050901).

Table 2.1: Recovery of AFB_1 spiked in aflatoxin-free sunflower seeds, cakes and crude oil sediments[a]

Sample type	Spiked AFB_1 Concentration (ng/g)	Mean AFB_1 Recovered (ng/g) ±SD	Recovery (%)
Sunflower seed meal	10	7.1±0.7	71
Sunflower seed meal	25	19.6±1.1	78
Sunflower seed cake	10	8.0±0.6	80
Sunflower seed cake	25	19.4±0.7	78
Sunflower crude oil sediments	25	24.8±0.5	99

[a] Values are means of three determinations; SD= Standard Deviation.

2.4.2 Concentrations of total aflatoxin in sunflower seeds in the year 2014 harvest season

Table 2.2 shows mean total aflatoxin concentrations in samples from various local oil extractors situated in Babati, Singida, Dodoma, Morogoro, Iringa, Mbeya and Karatu, the major hubs of sunflower processing in Tanzania. It also shows ranges of aflatoxin concentrations in the samples, the number of samples analyzed from each location, the number of samples found contaminated, and the percentage of contaminated samples. Dodoma, Babati, and Singida demonstrated higher average aflatoxin concentrations (59.6, 46.8, and 45.8 ng/g, respectively) than other towns

(Morogoro, 0.7 ng/g; Mbeya, 0.2 ng/g; and Karatu, 1.8 ng/g). The average aflatoxin concentrations in samples from Dodoma, Babati, and Singida also matched with their ranges (1.7–280.6, 1.8–162, and 1.4–261.8 ng/g, respectively), which were higher than other in towns (Morogoro, 1.6–1.9; 0–14; and 2.1–2.7 ng/g). Aflatoxins were not detected in samples collected from Iringa. Of seven samples from Dodoma, five were contaminated (71%), and one of the five samples had a high concentration of 280.6 ng/g. Of six samples from Singida, five were contaminated (83%), and one sample contained a maximum concentration of 262 ng/g while 83% of the six samples from Babati were contaminated, and the maximum level was 162 ng/g. Of seven samples from Mbeya, only one sample (14%) was contaminated (1.4 ng/g). Karatu had only four samples of which three were contaminated, and the maximum concentration was 2.7 ng/g.

Table 2.2: Aflatoxin concentrations in sunflower seeds collected from micro- and small-scale sunflower oil processors in Tanzania in the sunflower-harvesting season of 2014.

Location/Town	Number of samples	Number of positive samples and aflatoxin conc. (ng/g)	% of positive samples	Number of samples with conc. > 20 ng/g	% of samples with conc. >20 ng/g	Range of positive samples (min-max) (ng/g)	Median of positive samples (ng/g)	Mean (ng/g)
Babati-Manyara	6	5 [41.3, 1.8, n.d., 3.1, 73.0, 162.0]	83	3	50	1.8–162.0	41.3	46.8
Singida	6	5 [7.7, 1.4, n.d., 261.8, 1.9, 2.0]	83	1	17	1.4–261.8	2.0	45.8
Dodoma	7	5 [280.6, 32.3, 1.7, 48.9, 54.0, n.d., n.d.]	71	4	57	1.7–280.6	48.9	59.6
Morogoro	5	2 [n.d., 1.6, n.d., n.d., 1.9]	40	0	0	1.6–1.9	1.8	0.7
Iringa	7	n.d. [n.d., n.d., n.d., n.d., n.d., n.d., n.d.]	n.d.	0	0	0	0	n.d.
Mbeya	7	1 [n.d., n.d., 1.4, n.d., n.d., n.d., n.d.]	14	0	0	1.4	1.4	0.2
Karatu-Arusha	4	3 [2.1, 2.7, 2.4, n.d.]	75	0	0	2.1–2.7	2.4	1.8

Notes: n.d. = not detected (n.d.<LOD); LOD = limit of detection (1.4 ng/g).

2.4.3 Concentrations of total aflatoxin in sunflower seed cakes in the year 2014 harvest season

Sunflower seed cakes are the by-products of the ram pressing of sunflower seeds, and they are used to make animal feed. The cakes were collected from the same locations indicated in Table 2.2 above. The aflatoxin concentrations in sunflower cake samples are reported in Table 2.3. It should be noted that cake samples collected and reported in Table 2.3 were residues of seeds that were different from the seed samples reported in Table 2.2. Sunflower cakes from Dodoma and Mbeya had higher average aflatoxin concentrations (34 and 29 ng/g, respectively) than the other locations (Singida, 13; Morogoro, 11; Babati-Manyara, 6; Iringa, 3; and Karatu, 1.5 ng/g. The concentrations ranged from 1.9 to 88.2 and 2.8 to 97.7 ng/g in Dodoma and Mbeya, respectively. Singida (2.0–34.3), Morogoro (2.2–31.9), Babati (1.7–17.8), Iringa (1.7–5.3) and Karatu (1.5–2.2 ng/g) exhibited lower ranges of toxin contamination. The highest aflatoxin concentration (98 ng/g) was observed in one of the six samples collected from Mbeya. One out of seven samples from Dodoma were highly contaminated (89 ng/g). All cake samples from Dodoma, Singida, Babati and Iringa were contaminated while 80, 86 and 80% samples from Morogoro, Mbeya, and Karatu, respectively were contaminated. Locations where nearly all samples were contaminated but at aflatoxin concentrations below the action level of 20 ng/g were Karatu (2.2 ng/g), Iringa (5.3 ng/g) and Babati (17.8 ng/g). The maximum aflatoxin levels in Singida and Morogoro samples were 34.3 and 31.9 ng/g, respectively.

Table 2.3: Aflatoxin concentrations in sunflower seed cakes collected from micro- and small-scale sunflower oil processors in Tanzania in the sunflower-harvesting season of 2014.

Location/Town	Number of samples	Number of positive samples and aflatoxin conc. (ng/g)	% of positive samples	Number of samples with conc. > 20 ng/g	% of samples with conc. >20 ng/g	Range of positive samples (min-max) (ng/g)	Median of positive samples (ng/g)	Mean (ng/g)
Babati-Manyara	7	7 [1.7, 1.9, 2.1, 3.3, 17.8, 6.0, 9.0]	100	0	0	1.7–17.8	3.3	6.0
Singida	6	6 [17.9, 10.6, 10.3, 34.3, 4.2, 2.0]	100	1	17	2.0–34.3	10.5	13.2
Dodoma	7	7 [45.3, 46.3, 2.4, 46.8, 3.8, 88.2, 1.9]	100	4	57	1.9–88.2	45.3	33.5
Morogoro	5	4 [3.5, 16.2, 31.9, 2.2, n.d.]	80	1	20	2.2–31.9	9.9	10.8
Iringa	7	7 [2.6, 5.3, 1.8, 1.7, 3.7, 3.3, 2.1]	100	0	0	1.7–5.3	2.6	2.9
Mbeya	7	6 [2.8, n.d., 87.2, 7.8, 97.7, 3.0, 3.2]	86	2	29	2.8–97.7	5.5	28.8
Karatu-Arusha	5	4 [2.2, 1.5, n.d., 1.7, 2.2]	80	0	0	1.5–2.2	2.0	1.5

Notes: n.d. = not detected (n.d.<LOD); LOD = limit of detection (1.4 ng/g). These cake samples collected and reported in Table 2.3 are residues of seeds that were different from the seed samples reported in Table 2.2.

2.4.4 Concentrations of total aflatoxin in sunflower seeds in the year 2015 harvest season

Table 2.4 shows aflatoxin concentrations in sunflower seed samples collected in the sunflower-growing season of the year 2015. Morogoro, Singida, and Mbeya showed higher average concentrations (119 ng/g, 34 ng/g, and 21 ng/g, respectively) than Iringa (5.7 ng/g), Karatu (1.6 ng/g), Babati (1.2 ng/g) and Dodoma (0.5 ng/g). Three of the six samples from Morogoro were contaminated, and one sample (16.7%) showed a very high concentration (663 ng/g). Six of the seven samples from Singida were contaminated ranging from 1.6–217.6 ng/g and the highest concentration observed was 218 ng/g from one sample (14%) from this location. Eight of the nine samples from Mbeya were contaminated, and the highest concentration was 174 ng/g (range: 1.4–174 ng/g). Six of the seven samples from Iringa were contaminated (range: 1.5–28.6 ng/g). Dodoma had the lowest range (1.6–20 ng/g), and 29% (2/7) of the samples were contaminated. Three of the six samples from Karatu were contaminated (range: 1.9–3.7 ng/g).

Table 2.4: Aflatoxin concentrations in sunflower seeds collected from micro- and small-scale sunflower oil processors in Tanzania in the sunflower-harvesting season of 2015.

Location/Town	Number of samples	Number of positive samples and aflatoxin conc. (ng/g)	% of positive samples	Number of samples with conc. > 20 ng/g	% of samples with conc. >20 ng/g	Range of positive samples (min-max) (ng/g)	Median of positive samples (ng/g)	Mean (ng/g)
Babati-Manyara	7	4 [n.d., n.d., 2.3, n.d., 1.4, 1.9, 1.4]	57	0	0	1.4–2.3	1.7	1.2
Singida	7	6 [10.7, 1.6, n.d., 1.8, 217.6, 2.3, 2.6]	86	1	17	1.6–217.6	2.5	33.8
Dodoma	7	2 [n.d., n.d., n.d., n.d., n.d., 1.6, 2.0]	29	0	0	1.6–2.0	1.8	0.5
Morogoro	6	3 [46.3, 2.8, n.d., 662.7, n.d., n.d.]	50	2	33	2.8–662.7	46.3	118.6
Iringa	7	6 [2.4, 28.6, n.d., 1.5, 3.6, 2.6, 1.5]	86	1	14	1.5–28.6	2.5	5.7
Mbeya	9	8 [n.d., 2.5, 174.2, 1.4, 2.3, 1.9, 2.0, 3.3, 1.9]	89	1	13	1.4–174.2	2.2	21.1
Karatu-Arusha	5	3 [n.d., n.d., 1.9, 3.7, 2.3]	60	0	0	1.9–3.7	2.3	1.6

Notes: n.d. = not detected (n.d.<LOD); LOD = limit of detection (1.4 ng/g).

2.4.5 Concentrations of total aflatoxin in sunflower seed cakes in the year 2015 harvest season

The aflatoxin concentrations in sunflower seed cakes are listed in Table 2.5. Again, cake samples collected and reported in Table 2.5 were residues of seeds that were different from the seed samples reported in Table 2.4. Morogoro and Dodoma samples had higher average concentrations of 149.0 and 120.6 ng/g, respectively as compared to Singida (11.3 ng/g), Mbeya (7.1 ng/g), Babati (3.7 ng/g), Iringa (2.2 ng/g), and Karatu (1.8 ng/g). Based on their ranges, Dodoma (13.3–598.4 ng/g), Morogoro (2.7–536 ng/g), Mbeya (1.4–20.3 ng/g), and Singida (3.2–52.8 ng/g) were the only locations that showed maximum concentrations in their cakes above the action level of 20 ng/g. Mbeya, Iringa, Karatu, and Babati had lower ranges of 1.4–20.3, 1.5–12, 1.7–11.2, and 1.5–13.8 ng/g in cakes, respectively; while 100% of the samples from Morogoro were contaminated with very high aflatoxin concentrations with a maximum concentration of 536.0 ng/g. Fifty-seven percent of the samples from Dodoma were contaminated with a maximum concentration of 598.4 ng/g.

Table 2.5: Aflatoxin concentrations in sunflower seed cakes collected from micro- and small-scale sunflower oil processors in Tanzania in the sunflower-harvesting season of 2015.

Location	Number of samples	Number of positive samples and aflatoxin conc. (ng/g)	% of positive samples	Number of samples with conc. > 20 ng/g	% of samples with conc. >20 ng/g	Range of positive samples (min-max) (ng/g)	Median of positive samples (ng/g)	Mean (ng/g)
Babati-Manyara	7	5 [n.d., 13.8, 1.5, 2.1, n.d., 1.5, 6.8]	71	0	0	1.5–13.8	2.1	3.7
Singida	7	4 [n.d., 15.7, 3.2, n.d., 52.8, n.d., 7.1]	57	1	14	3.2–52.8	11.4	11.3
Dodoma	7	4 [111.0, 121.2, n.d., n.d., n.d., 598.4, 13.3]	57	3	43	13.3–598.4	116.1	120.6
Morogoro	6	6 [229.4, 40.9, 2.7, 536.0, 10.1, 74.7]	100	4	67	2.7–536.0	57.8	149.0
Iringa	7	3 [n.d., 12.0, n.d., n.d., n.d., 1.5, 1.9]	43	0	0	1.5–12.0	1.9	2.2
Mbeya	9	9 [1.4, 7.5, 5.0, 4.9, 3.2, 1.5, 3.2, 17.1, 20.3]	100	0	0	1.4–20.3	4.9	7.1
Karatu-Arusha	5	2 [n.d., 1.7, n.d., n.d., 11.2]	40	0	0	1.7–11.2	6.5	1.8

Notes: n.d. = not detected (n.d.<LOD); LOD = limit of detection (1.4 ng/g). These cake samples collected and reported in Table 2.5 are residues of seeds that were different from the seed samples reported in Table 2.4.

2.4.6 Concentrations of total aflatoxin in sediments from crude sunflower oils collected in the year 2014 harvest season

Aflatoxin was not detected in sediments of all sunflower crude oil samples from Babati-Manyara. Only one out of six sediment samples from Singida had the detectable level of aflatoxin (6.5 ng/g). Of seven out of sediment samples from Dodoma, only one sample was contaminated (3.8 ng/g). Likewise, Morogoro had only one sample contaminated (5.0 ng/g) amongst four sediment samples (Table 2.6). The ranges of contamination were Singida (0.0–6.5), Dodoma (0.0–3.8), and Morogoro (0.5–5.0 ng/g).

2.4.7 Concentrations of total aflatoxin in sediments from crude sunflower oils collected in the year 2015 harvest season

Aflatoxin was not detectable in sediments from crude sunflower oils from Babati-Manyara, Singida, and Dodoma. However, Morogoro showed five of six contaminated sediment samples. Of these, two had concentrations (85.3 and 42 ng/g) that exceeded the regulatory limit of 20 ng/g. The mean contamination level of 25.2 ng/g in this location also exceeded the FDA action level. The range of aflatoxin concentration in Morogoro was 2.4–85.3 ng/g (Table 2.7).

Table 2.6: Aflatoxin concentrations in sediments of crude sunflower oils collected from micro- and small-scale sunflower oil processors in Tanzania in the sunflower-harvesting season of 2014.

Location/Town	Number of samples	Number of positive samples and aflatoxin conc. (ng/g)	% of positive samples	Number of samples with conc. > 20 ng/g	% of samples with conc. >20 ng/g	Range of positive samples (min-max) (ng/g)	Median of positive samples (ng/g)	Mean (ng/g)
Babati-Manyara	6	0 [n.d., n.d., n.d., n.d., n.d., n.d.]	0	0	0	0	0	0
Singida	6	1 [n.d., n.d., 6.5, n.d., n.d., n.d.]	17	0	0	0.0–6.5	6.5	1.1
Dodoma	7	1[3.8, n.d., n.d., n.d., n.d., n.d., n.d.]	14	0	0	0.0–3.8	3.8	0.5
Morogoro	4	1[n.d., n.d., n.d., 5.0]	25	0	0	0.5–5.0	5.0	1.3

Notes: n.d. = not detected (n.d.<LOD); LOD = limit of detection (1.4 ng/g). These sediment samples are residues in the crude sunflower oils collected from micro-scale oil processors. They may not necessarily originate from the same seed and cakes presented in Tables 2.2 and 2.3.

Table 2.7: Aflatoxin concentrations in sediments of crude sunflower oils collected from micro- and small-scale sunflower oil processors in Tanzania in the sunflower-harvesting season of 2015.

Location/Town	Number of samples	Number of positive samples and aflatoxin conc. (ng/g)	% of positive samples	Number of samples with conc. > 20 ng/g	% of samples with conc. >20 ng/g	Range of positive samples (min-max) (ng/g)	Median of positive samples (ng/g)	Mean (ng/g)
Babati-Manyara	7	0 [n.d., n.d., n.d., n.d., n.d., n.d., n.d.]	0	0	0	0	0	0
Singida	7	0 [n.d., n.d., n.d., n.d., n.d., n.d., n.d.]	0	0	0	0	0	0
Dodoma	7	0 [n.d., n.d., n.d., n.d., n.d., n.d., n.d.]	0	0	0	0	0	0
Morogoro	6	5 [2.4, n.d., 85.3, 8.7, 41.7, 12.9]	83	2	33	2.4–85.3	12.9	25.2

Notes: n.d. = not detected (n.d.<LOD); LOD = limit of detection (1.4 ng/g). These sediment samples are residues in the crude sunflower oils collected from micro-scale oil processors. They may not necessarily originate from the same seed and cakes presented in Tables 2.4 and 2.5.

2.4.8 Exposure assessment and risk characterization for humans

Table 2.8 reports the estimates of dietary exposures and population risk for primary liver cancer cases from consumption of contaminated sunflower seeds in seven locations of Tanzania for sunflower seasons of 2014 and 2015. The dietary exposure estimates of 25 and 21 ng/kgbw/day appeared to be the highest for contaminated sunflower seeds collected in 2014 from local oil processors based in Dodoma and Manyara, respectively. These exposures revealed population risks for primary liver cancer cases of 0.91 and 0.77 cases per year per 100,000 individuals, respectively for Dodoma and Manyara. The rest of the locations assessed in 2014 showed lower exposures ranging from 0.0 to 1.2 ng/kgbw/day, and lower cancer risks ranging from 0.00 to 0.04 cancer cases per year per 100,000 individuals (Table 2.8). For sunflower seeds collected in 2015, the assessment indicated that Morogoro had the highest exposure estimate of 23.8 ng/kgbw/day, and population risk for cancer of 0.86 cancer cases per year per 100,000 amongst seven locations assessed. The rest of the locations assessed in 2015, showed lower exposures ranging from 0.9 to 1.3 ng/kgbw/day, and lower cancer risks ranging from 0.03 to 0.05 cancer cases per year per 100,000 individuals.

Table 2.9 presents the estimates of dietary exposures and population risk for primary liver cancer cases from consumption of contaminated crude sunflower oils collected in 2014 and 2015 in Manyara, Singida, Dodoma, and Morogoro. Generally, in 2014 sunflower season the dietary exposures ranged from 0.0 to 2.4 ng/kgbw/day, and population risk for liver cancer cases ranged from 0.00 to 0.09 cancer cases per year per 100,000 individuals. In 2014, the highest exposure (2.4 ng/kgbw/day) and population risk for liver cancer (0.09 cancer cases per year per 100,000 individuals) appeared in Singida crude oils. In contrast, in 2015 sunflower season there were no observed dietary exposures and no risk of liver cancer in Manyara, Singida, and Dodoma.

Morogoro showed high exposure of 3.6 ng/kgbw/day, and population risk for liver cancer of 0.13 cancer cases per year per 100,000 individuals.

Table 2.8: Possible human exposure to total aflatoxin and risk characterization as a result of consumption of sunflower seeds collected from micro- and small-scale sunflower oil processors in Tanzania in the sunflower-harvesting season of 2014 and 2015.

	2014				2015			
Location/Town	Median contamination level (ng/g)	Daily intake per person per day in Urban* (g/day)	Exposure (ng/kg bw/day)**	Population risk for primary liver cancer (cancers/year per 100,000)**	Median contamination level (ng/g)	Daily intake per person per day in Urban* (g/day)	Exposure (ng/kg bw/day)**	Population risk for primary liver cancer (cancers/year per 100,000)**
Babati-Manyara	**41.3**	36	**21.2**	**0.77**	1.7	36	0.9	0.03
Singida	2.0	36	1.0	0.04	2.5	36	1.3	0.05
Dodoma	**48.9**	36	**25.2**	**0.91**	1.8	36	0.9	0.03
Morogoro	1.8	36	0.9	0.03	46.3	36	**23.8**	**0.86**
Iringa	0.0	36	0.0	0.00	2.5	36	1.3	0.05
Mbeya	1.4	36	0.7	0.03	2.2	36	1.1	0.04
Karatu-Arusha	2.4	36	1.2	0.04	2.3	36	1.2	0.04

Notes: n.d. = not detected (n.d.<LOD); LOD = limit of detection (1.4 ng/g). *Estimated amount of seeds an adult assumed to weigh 70kg consumes per day living in urban areas in Tanzania. The daily intake of 36 g of sunflower seeds consumed by an individual per day was obtained from a household of 2 people living in Kinondoni–Dar es Salaam, Tanzania who consumed a total of 0.5kg per seven days. (Data source: Tanzania National Panel survey 2011; http://catalog.ihsn.org/index.php/catalog/4617). Thus, the calculations are based on the assumption that this daily intake is applicable to all towns investigated in this study whose values of daily intake per individual could not be obtained **Values obtained using equations adapted from Shephard (2008).

Table 2.9: Possible human exposure to total aflatoxin and risk characterization as a result of consumption of crude oils collected from micro- and small-scale sunflower oil processors in Tanzania in the sunflower-harvesting season of 2014 and 2015.

	2014				2015			
Location/Town	Median contamination level (ng/g)	Daily intake per person per day in Urban* (g/day)	Exposure (ng/kg bw/day)**	Population risk for primary liver cancer (cancers/year per 100,000)**	Median contamination level (ng/g)	Daily intake per person per day in Urban* (g/day)	Exposure (ng/kg bw/day)**	Population risk for primary liver cancer (cancers/year per 100,000)**
Manyara-Babati	0.0	19.2	0.0	0.00	0.0	19.2	0.0	0.00
Singida	6.5	26.1	2.4	0.09	0.0	26.1	0.0	0.00
Dodoma	3.8	14.0	0.8	0.03	0.0	14.0	0.0	0.00
Morogoro	5.0	19.4	1.4	0.05	12.9	19.4	**3.6**	**0.13**

Notes: n.d. = not detected (n.d.<LOD); LOD = limit of detection (1.4 ng/g). Total aflatoxin was extracted from the sediments of the crude sunflower oils, which are normally consumed unrefined. The sediments are solid particle residues in the crude sunflower oils collected from micro-scale oil processors. *Estimated amount of vegetable oil an adult assumed to weigh 70kg consumes per day living in urban areas in Tanzania (Data source: Tanzania National Panel survey 2011; http://catalog.ihsn.org/index.php/catalog/4617). **Values obtained using equations adapted from Shephard (2008).

2.5 Discussion

Sunflower is an important oilseed crop in Tanzania that contributes about 36% of the total cooking oil consumed in the country each year (RLDC 2008). Besides cooking oil, sunflower seeds are a source of sunflower cakes used for dairy and beef cattle and poultry feedstuffs. Tanzanians also consume roasted and raw sunflower seeds as a food snack. However, there are no reports on potential aflatoxin contamination of sunflower seeds and cakes grown in Tanzania. The available aflatoxin reports focused mainly on aflatoxin contamination of agricultural commodities such as maize, cassava, and market-cured fish (Kimanya et al. 2008; Manjula et al. 2009; Mugula and Lyimo 1992). Several reports outside Tanzania (Dawar and Ghaffar 1991a, b; Jiménez et al. 1991; Beheshti and Asadi 2013) indicate that *A. flavus* and *A. parasiticus* can infect sunflower and cause aflatoxin accumulation in seeds and cakes. The present study aimed at determining aflatoxin levels in seed and cake samples collected from micro- and small-scale sunflower oil processors in Babati, Singida, Dodoma, Morogoro, Iringa, Mbeya and Karatu towns of Tanzania.

To investigate the recurring nature of aflatoxin contamination in sunflower seed and cakes, two surveys were carried out across the country in two consecutive years. The incidences of aflatoxin contamination and aflatoxin concentrations in some individual samples were quite high in both harvest seasons.

The aflatoxin concentrations in the first survey are shown in Tables 2.2 and 2.3 for seed and cake samples, respectively. Dodoma had high aflatoxin concentrations in several samples (32.3, 48.9, 54 and 280.6 ng/g) all of which were above the action level of 20 ng/g. Its neighboring towns, Babati and Singida, also had samples contaminated with unacceptable aflatoxin concentrations of 41.3, 73, 162 and 261.8 ng/g, respectively. Except Mbeya, the

incidence of aflatoxin contamination in sunflower cakes (Table 2.3) appeared to correlate with that of sunflower seeds in this survey. Dodoma had the highest aflatoxin concentrations in sunflower cakes (45, 46, 47 and 88 ng/g), while Singida had only one extreme contamination event (34 ng/g) of six contaminated samples and Mbeya had two extremes (87 and 98 ng/g) of the seven samples tested.

These results demonstrate that sunflower samples collected from June to August 2014 were infected with aflatoxin-producing fungi and many samples particularly from Singida, Dodoma, Morogoro and Babati were contaminated with high aflatoxin concentrations above the allowable regulatory limit of 20 ng/g (Tables 2.2, 2.3, 2.4 and 2.5). The contamination of Tanzanian sunflower seeds may have originated in the field and carried over to the storage facilities before processing. Dodoma and Singida are in lowland central Tanzania, characterized by semi-arid, warm and drought climate, which are favorable environments for aflatoxigenic molds to grow (Cotty and Jaime-Garcia 2007). The sunflower cultivation months in Tanzania are usually January through May and the meteorological data (Figure 2.1) indicate that in this period of the year 2014, Dodoma and Singida had received less rainfall than other regions, which was indicative of drought.

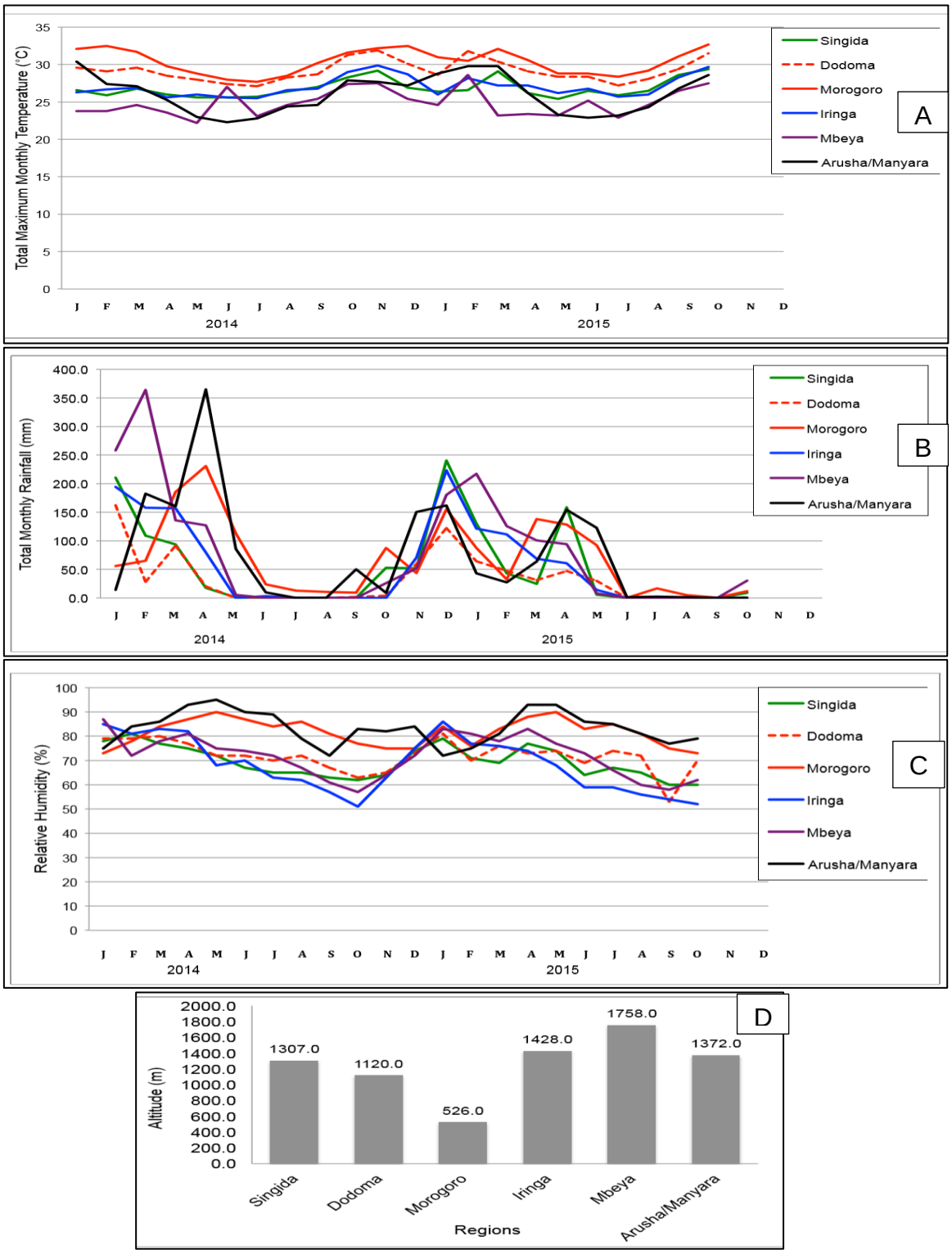


Figure 2.1: Meteorological data showing maximum monthly mean temperature (˚C) [A]; total monthly rainfall (mm) [B]; monthly mean relative humidity (%)[C]; and region altitude (m)[D]. Source: Tanzania Meteorological Authority (TMA), Ubungo Plaza, 3rd Floor, P.O.Box 3056, Dar es Salaam, United Republic of Tanzania. They were obtained in 2015.

Climatic conditions significantly influence the distribution, density, and the structure of *A. flavus* and *A. parasiticus* communities and susceptibility of crop plants to such communities (Cotty and Jaime-Garcia 2007; Donner et al. 2009; Probst et al. 2014). Drought causes stress to plants in the field, thus rendering them more susceptible to fungal infestation and insect damage, and exacerbating aflatoxin accumulation in seeds (Magan et al. 2003; Magan 2015). In contrast, the cooler climatic conditions, higher altitude, and more rainfall in Iringa, Mbeya, Manyara and Karatu (Tables 2.2, 2.3 and Figure 2.1 *vide supra*) may have advantageously led to relatively low aflatoxin concentrations. However, poor storage facilities and poor post-harvest handling of seeds and cakes are also major factors for increased aflatoxin accumulation (Magan et al. 2003; Khlangwiset and Wu 2010). Thus, the findings for Dodoma and Singida could be attributed to both drought and poor post-harvest handling of the seeds and cakes. The dry seeds entering the storage facility from the field may have microflora and insects, which introduce respiratory activity and raise moisture content of the seeds. Consequently, molds carried over in seeds from the field to the storage facilities continue to grow and accumulate aflatoxin in the seeds before processing. This may account for the observed higher aflatoxin concentrations (87 and 98 ng/g) in a few sunflower cake samples collected from Mbeya (Table 2.3) and (41, 73, and 162 ng/g) in a few seed samples from Manyara (Table 2.2) despite their cooler climate and sufficient rainfall (Figure 2.1B). Although Morogoro is characterized by high temperature and relative humidity (Figure 2.1A & 2.1C), the adequate rainfall (Figure 2.1B) it received in year 2014 could have alleviated the severity of fungal contamination and aflatoxin levels. If Morogoro had received less rainfall, perhaps the extent of aflatoxin contamination would have been similar to that of Dodoma as these regions are contiguous. This was the case in 2015 (Figure 2.1B, Tables 2.4 and 2.5).

The preliminary findings in 2014 prompted us to carry out another survey in 2015 to investigate the recurrence of aflatoxin contamination in sunflower seeds and cakes in the same towns where samples were collected in 2014. Tables 2.4 and 2.5 indicate the aflatoxin concentrations observed in samples collected from such towns. Overall, there was more aflatoxin contamination of sunflower products in 2015. The highest aflatoxin concentration in sunflower seeds was observed in one sample collected from Morogoro (663 ng/g), and one sample from Singida (218 ng/g). Some Morogoro sunflower cakes were also highly contaminated (41, 75, 229, and 536 ng/g). While Dodoma demonstrated a high concentration (281 *n*g/g) in one of its sunflower seed samples in 2014, it had lower concentrations in 2015 (Table 2.4). However, three cake samples were highly contaminated in 2015 (111, 121, and 598 ng/g; Table 2.5). It is possible that certain highly contaminated seeds were not sampled, and contributed to the higher aflatoxin levels found in the cakes. The overall incidence of aflatoxin contaminations was much higher in sunflower seeds and cakes harvested in 2015 than in 2014.

Meteorological data (Figure 2.1) indicated that the whole Tanzanian sunflower corridor received less rain in 2015 (higher aflatoxin contamination overall) than in 2014, which suggests that conditions were favorable for pre-harvest aflatoxin contamination. Another factor that might account for higher aflatoxin contamination in year 2015 was storage duration of the samples collected. While the samples collected in year 2014 had stayed in storage for 3 months (May–July 2014) prior to sample collection, a subset of samples collected in 2015 had been in storage for 5 months (May–September 2015). In addition, the semi-arid climatic conditions in Central Tanzania (Dodoma, and Singida) and neighboring regions (Manyara and Morogoro) might have contributed to the observed levels, because these conditions are conducive to aflatoxigenic molds (Cotty and Jaime-Garcia 2007; Magan et al. 2011; Magan 2015; Mayaya et al. 2015).

The current problem of climate change may increase the risk of aflatoxin contamination in susceptible crops in the near future (Cotty and Jaime-Garcia 2007; Donner et al. 2009; Wu et al. 2011; Probst et al. 2014). *A. flavus* and *A. parasiticus* distribution is predicted to increase with global warming (Paterson and Lima 2010). Drought, high humidity and high temperature cause stress to plants and reduce production of phytoalexins, the chemicals used by plants to resist fungal infection (Wotton and Strange 1987). When such conditions prolong, crop plants in the field weaken and are rendered susceptible to fungal infection (Hill et al. 1983; Cotty and Jaime-Garcia 2007). Mayaya et al. (2015) reported that Dodoma and a large part of Central Tanzania were often characterized by drought due to inadequate, unpredictable rainfall and increase in temperature. Deforestation, wild fires and charcoal production on Uluguru mountains in Morogoro have resulted in gradual micro-climate change manifested by lower precipitation and higher temperatures (Paavola 2008). This may likely expand the ecological environment of aflatoxigenic molds in Morogoro as well.

Like seeds, cakes are vulnerable to aflatoxin contamination. In the two surveys, 80% of 92 sunflower cake samples were contaminated with aflatoxin ranging from 1.4–598.4 *n*g/g, compared to only 59% of 90 sunflower seed samples contaminated with aflatoxin ranging from 1.4–662.7 ng/g illustrating that more cake samples were contaminated than seeds. This may be because aflatoxin contamination is concentrated in a small number of seeds, which may have not been sampled; but when the highly contaminated seeds are ground into cakes, aflatoxin becomes detectable. For example, aflatoxin could not be detected in sunflower seed samples from Iringa, but was detected in its sunflower cake samples (mean = 2.9 ng/g and range = 1.7–5.3 ng/g) although in low levels (Table 2.2 and 2.3). Additionally, since the cakes and seeds were stored in the same storage warehouse, cross-contamination of spores from the seeds and the floor of

storage facility was probable. The degree of sanitation, ventilation, and sun drying duration varied from one oil mill to another; which could also account for the observed variability in aflatoxin concentrations in their sunflower seeds and cakes collected in one location (Tables 2.2, 2.3, 2.4, 2.5).

These surveys were not without a challenge. It was hard to sample static sacks situated in the interior of sack piles in the stores (Whitaker 2006). We obtained samples only from the sacks peripherally located in each store. The small sample size per region was also a limitation. Large sample sizes would provide more information on the incidence of aflatoxin contamination and better check great variability in aflatoxin levels from one region to another. We therefore recommend that future survey studies should address these limitations. However, despite this challenge, the results still indicated that incidence of aflatoxin contamination in sunflower seeds and cakes from central Tanzania was quite high.

The results for seven locations of Tanzania (Tables 2.2, 2.3, 2.4, and 2.5) corroborate reports from Spain (Jiménez et al. 1991), India (Bhat 1988; Banu and Muthumary 2005), Iran (Beheshti and Asadi 2013), Pakistan (Dawar and Ghaffar 1991b) and Sudan (Elzupir et al. 2010), which indicated that sunflower seeds and cakes were susceptible to aflatoxin contamination. The high-aflatoxin samples obtained from Dodoma, Morogoro, Babati and Singida towns may have resulted from a combination of drought and sub-optimal postharvest handling and storage of seeds and cakes (Cotty and Jaime-Garcia 2007; Paavola 2008; Mayaya et al. 2015). Anecdotal information from the sunflower oil processors (buyers of the sunflower seeds) revealed that unscrupulous sellers might adulterate sound seeds with moldy seeds to increase the weight of the packed sunflower seed bags for economic profit. Unfortunately, smaller quantities of moldy

seeds in the midst of sound seeds may serve as reservoirs for increasing aflatoxin contamination in storage.

Climatically, Eastern and Central province of Kenya and Central regions of Tanzania may have similar semi-arid conditions, which are favorable for the S-strain of *A. flavus* implicated with the Eastern and Central Kenya aflatoxicosis outbreak in 2004 (Lewis et al. 2005; Probst et al. 2014). However, prevalence of S-strain in Central Tanzania is not known and it is a potential gap for future study.

Partially roasted and salted sunflower seeds are primarily consumed as snacks in Tanzania. Also, crude sunflower oils are consumed as vegetable cooking oils and in Tanzania crude sunflower oils are more likable than the refined oils due to the common conviction that they are rich in nutrients and do not contain additives. Although previous reports have shown that sunflower seeds (Dawar and Ghaffar 1991) and crude sunflower oils (Elzupir et al. 2010) are not immune to aflatoxin contamination, no reports on aflatoxin risk assessment for consumption of such products are available in Tanzania. Therefore, this risk assessment to our knowledge is the first report to be produced in this region. The results of exposure estimates and population risks for primary liver cancer cases for these sunflower seasons of 2014 and 2015 are evident that seeds from Dodoma, Manyara, and Morogoro processors (Table 2.8) were not safe. The translation of these results is that in each of these three towns, approximately one adult per 100,000 is likely to die of primary liver cancer each year as a result of daily consumption of 36 g of contaminated sunflower seeds. Considering major dietary exposures to aflatoxin through maize, cassava and peanuts consumed as main courses, exposure through sunflower seeds consumed as snacks, may be supplementary thus, augmenting the extent to which such populations are chronically exposed to total aflatoxin. Our survey and risk assessment data in

conjunction with local meteorological data (Figure 2.1) somewhat suggest that climatic conditions (drought) could be a substantial risk factor for higher accumulations of aflatoxin in sunflower seeds in Dodoma, Singida, Manyara, and Morogoro.

Because seeds from Dodoma, Singida, Manyara, and Morogoro were not safe due to extremely high contamination levels (Tables 2.2 and 2.4) and relatively higher risk assessment data (Table 2.8), we selected the crude sunflower oil samples from these towns to obtain crude oil sediments from which we analyzed total aflatoxin. Interestingly, except Morogoro oil sediments of 2015, none of the sediments from the crude oils of all towns showed high risk (Table 2.9), suggesting that the crude sunflower oils from these towns in 2014 and 2015 were safe. However, the fact that many oil sediments from Morogoro were contaminated indicates that unrefined sunflower oil can be susceptible to aflatoxin contaminations. One important lesson from Morogoro data of sunflower seed contamination levels and the sediment data in 2015 is that the higher the levels of total aflatoxin in the seeds, the greater the likelihood of having aflatoxin in the sediments of the crude oils.

Parker and Melnick (1966) reported that oil refining eliminates aflatoxin from the contaminated crude oils. However, it was evident during our surveys that the micro-scale sunflower oil processors in Tanzania are unable to afford elaborate refinery plant installations. Instead, they use low-tech filter systems to clarify the crude oils that do not remove the sediments completely. The aflatoxin levels in the sediments from Morogoro crude oils are an illustration of this point of view, and they are an alert that individuals consuming unchecked crude oils may be potentially at risk of chronic exposure to aflatoxins.

Since the liver cancer risk in Dodoma municipal for the sunflower seed consumption in 2014 was estimated to be 0.9 cases per year per 100,000 adults, then four persons per 410,956

total population (Tanzania–2012 Population and Housing Census) of this location are likely to die of aflatoxin-induced liver cancer annually should the contamination trend persist in the same fashion. Similarly, because liver cancer risk in Morogoro was estimated to be 0.86 cases per year per 100,000, then three persons per 315,866 are likely to die of aflatoxin-induced liver cancer as a result of sunflower seed consumption should the aflatoxin contamination trend persist in the same fashion. It should be noted that these death estimations assumed that the all the populations were adults without considering their sex and age. In Tanzania, the maximum dietary aflatoxin exposure of 50 ng/kgbw/day through maize and peanut consumption estimated by Liu and Wu (2010) appear to be twice as much greater than the maximum exposure of 25.2 ng/kgbw/day through sunflower seed consumption estimated by us. Deductively, maize and peanuts are the primary staples and that the daily intakes and susceptibility to aflatoxin producers must be higher than sunflower seeds. Nonetheless, our dietary exposure data strongly suggest sunflower seed consumption can also contribute significantly to dietary exposure to aflatoxin. Broadly, sunflower seeds can be as vulnerable to aflatoxin accumulation in stores as maize and peanuts. Also, exposure data for central Tanzania are a reminder that this region is more susceptible to aflatoxin contamination, and that interventions are required to prevent probable aflatoxicosis in the area.

As for oil sediments, aflatoxin levels were not of great concern suggesting that there may be limited solubility of aflatoxins into the crude oil. However, refining of the crude oils should be encouraged to guarantee complete elimination of aflatoxin from the sunflower cooking oil. As pointed out earlier, contaminations found in the oil sediments from Morogoro in 2015 are evidence that oil clarification using fabric filters alone is not sufficient to guarantee complete

elimination of aflatoxin from the crude oils particularly if the crude oil produced originated from heavily contaminated sunflower seeds.

2.6 Conclusions

To summarize: In our study, 59% of 90 sunflower seed samples and 80% of 92 cake samples collected from sunflower oil processors across Tanzania were contaminated with aflatoxins. Moreover, 14% of seed samples and 17% of cake samples total were contaminated with aflatoxin concentrations above allowable limit of 20 *n*g/g, with several samples having levels in several hundred ng/g of aflatoxin. The sediments of crude sunflower oils from Morogoro collected in 2015 were found contaminated. The sediments of all other crude oils from Dodoma, Singida, and Babati-Manyara were safe for human consumption.

Our risk assessment that used assumptions, median contamination levels of total aflatoxin, and combined cancer potency factor congruent with the HBV prevalence rate in Tanzania, provides an insight into the magnitude of the risk. It has estimated the dietary exposures to total aflatoxin and population risk of liver cancer for humans as a result of consuming contaminated sunflower seeds, and unrefined oils from various locations of Tanzania. The dietary exposure estimates and population risks for primary liver cancer cases computed from were higher in central Tanzania as compared to other locations. These data lay a platform for processors and local authorities to implement the intervention strategies, especially in storage, to prevent and control aflatoxin contamination along the sunflower commodity value chain, to enhance food safety in Tanzania.

CHAPTER 3: BIOACTIVE COMPOUNDS IN *DIOSPYROS MAFIENSIS* ROOTS INHIBIT GROWTH, SPORULATION AND AFLATOXIN PRODUCTION BY *ASPERGILLUS FLAVUS* AND *ASPERGILLUS PARASITICUS*

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3.1 Abstract

Diospyros mafiensis F. White is a medicinal shrub or small tree (6 m tall) widely distributed in the Zanzibar-Inhambane regional mosaic and traditionally used to treat leprosy, diarrhea, and skin fungal infections in Tanzania and Mozambique. The objective of the current study was to determine the anti-aflatoxigenic properties of compounds from *D. mafiensis* root bark against vegetative growth, sporulation and aflatoxin production by *Aspergillus flavus* and *Aspergillus parasiticus*. Our methods involved bioassay-guided extraction, fractionation, and isolation of bioactive compounds using *A. parasiticus* B62 were employed. The bioactive compounds were elucidated using ^{1}H and ^{13}CNMR and LC-MS experiments. Growth inhibition was determined by measuring the colony diameter of *A. flavus* AF3357 and *A. parasiticus* SU-1 ATCC56775. Inhibitory effects on sporulation were estimated using a hematocytometer. Total aflatoxin was quantified by direct competitive enzyme-linked immunosorbent assay (ELISA). The bioactive compounds Diosquinone (DQ) and 3-Hydroxydiosquinone (3HDQ) were identified. DQ weakly inhibited *A. flavus* and *A. parasiticus* vegetative growth (MIC_{50} >100 µg/mL) and 3HDQ strongly inhibited *A. flavus* (MIC_{50} = 14.9 µg/mL) and *A. parasiticus* (MIC_{50} = 39.1 µg/mL). DQ strongly reduced total aflatoxin production by *A. flavus* from 157 to 36 ng/plate, and by *A. parasiticus* from 1145 ng/plate to 45 ng/plate at 100 µg/mL. 3HDQ reduced total aflatoxin production by *A. parasiticus* from 1145 to 32 ng/plate; stimulated production by *A. flavus* from 157 to 872 ng/plate at 12.5 µg/mL but reduced to 45 ng/plate at 100 µg/mL. In conclusion, DQ

and 3HDQ could be used as natural antifungal compounds to prevent mold growth and aflatoxin accumulation in food and feed.

3.2 Introduction

Aspergillus flavus and *Aspergillus parasiticus* produce toxic secondary metabolites called aflatoxins, which have deleterious health effects to humans and animals that include immunosuppression (Jiang et al. 2008), growth impairment (Khlangwiset et al. 2011), aflatoxicosis (Strosnider et al. 2006) and liver cancer (Liu and Wu 2010). The World Health Organization (2008) estimated that between 25,000 and 155,000 people die each year of liver cancer linked to chronic exposure to aflatoxins through contaminated food, and over 83% of such deaths occur in Sub-Saharan African countries (Liu and Wu 2010; Strosnider et al. 2006). Unfortunately, severe fungal deterioration and contamination of food occur during storage due to conditions favorable to fungal growth (Hell at al. 2000). Additionally, chronic exposure to *A. flavus* and *A. parasiticus* spores is now known to cause a respiratory disease called allergic bronchopulmonary aspergillosis (ABPA). Although ABPA occurs relatively infrequently, it can be deadly particularly for immunocompromised individuals (Denning et al. 2013). Denning and coworkers (2013) calculated an estimate of 389,900 cases of ABPA associated with invasive fungal infections is likely in Africa. Thus, individuals winnowing infected seeds during harvesting or from granaries without dust masks are likely to inhale fungal spores and thus, may be at risk of ABPA and invasive aspergillosis (Pfaller et al. 2016), which are often accompanied with chronic asthma (Denning et al. 2013).

In recent years, there have been growing concerns associated with the indiscriminate use of synthetic pesticides for crop protection against the molds in storage. The concerns associated with synthetic pesticides include fungal resistance, toxicological effects on consumers, non-

biodegradability, and prohibitive costs (da Cruz Cabral et al. 2013). As an alternative to synthetic pesticides, medicinal plants may be useful sources of naturally-occurring, biodegradable, readily available, and inexpensive food preservatives that could be useful to prevent growth, sporulation and aflatoxin production by aflatoxigenic molds in food during storage (Bluma et al. 2008; Bluma and Etcheverry 2008; Velazhahan et al. 2010; El-Nagerabi et al. 2013; Alejandra et al. 2013; Kedia et al. 2014; and Prakash et al. 2014). These studies showed that exposure of *A. flavus* and *A. parasiticus* to medicinal plant extracts or pure compounds significantly reduced or completely inhibited their growth and toxin production. Additionally, because such extracts contain a variety of compounds, their synergistic modes of action against molds may reduce the likelihood of development of resistance unlike that related to use of individual synthetic pesticides (da Cruz Cabral et al. 2013).

The genus *Diospyros* of the family Ebenaceae has over 350 species of economically important medicinal plants widely distributed in tropical countries worldwide (Mallavadhani et al. 1998). All plant parts (leaves, stem bark, root bark, fruits, and seeds) of *Diospyros* species have a myriad of biologically active compounds against viruses, bacteria, fungi, and termites (Marston et al. 1984; Mallavadhani et al. 1998). The unique medicinal properties of *Diospyros* species have contributed to their use as traditional medicines in tropics and subtropics to treat human ailments such as gonorrhea and tuberculosis in Cameroon (Kuete et al. 2009), asthma in Sri Lanka (Herath et al. 1978), leprosy in Tanzania (Khan et al. 1980), schistosomiasis in Malawi (Gafner et al. 1987), and of particular importance to the current work, fungal infections in Tanzania (Hamza et al. 2006).

Tanzanian *Diospyros* species are widely distributed in the Zanzibar–Inhambane regional mosaic and are abundant and endemic to this region (White 1988). The natives in this area have

been using this plant in many ways ranging from a source of material for house construction, edible fruits (Hall and Rodgers 1986; White 1988) to traditional medicines for treating human diseases (Khan et al. 1980). The leaves, root and stem barks of *Diospyros* species have been used in this region by traditional healers since olden times to prepare infusions, decoctions, root powders, and topical herbs for treating human ailments. Such illnesses include ulcers, dysentery, leprosy, whooping cough, general body weakness, non-insulin diabetes mellitus and oral candidiasis (Watt and Breyer-Brandwijk 1962; Moshi and Mbwambo 2002; Hamza et al. 2006). Also, its termicidal property makes poles derived from the tree preferable for the construction of durable houses (Hall and Rodgers 1986). However, *Diospyro*s species grown in this region have not been studied for their inhibitory activity against vegetative growth, sporulation and aflatoxin production by aflatoxigenic molds such as *A. flavus* and *A. parasiticus*. The objective of the present work was to study the anti-aflatoxigenic activity of *D. mafiensis* root extracts from Tanzania against *A. flavus* and *A. parasiticus* as part of the search for safer natural antimycotics that could be used to protect stored food crops.

3.3 Materials and methods

3.3.1 General experimental procedures

All solvents used for isolation and purification were of ACS reagent grade (Sigma-Aldrich Chemical Co., St. Louis, MO, USA). Merck silica gel (60 mesh size, 35−70 μm) with a particle size of 60 μm was used for preparative medium-pressure liquid chromatography (MPLC). Silica gel plates (250 μm; Analtech, Inc., Newark, DE, USA) were used for preparative thin-layer chromatography (TLC) and developed plates viewed using ultraviolet light at 254 or 366 nm using a Spectroline CX-20 ultraviolet fluorescence analysis cabinet (Spectroline Corp., Westbury, NY, USA). After viewing and locating spots under UV light, plates were sprayed with 10% sulfuric acid solution in water and charred to observe spots that were not visible under UV. NMR spectra were recorded on a 500 MHz (Varian Unity ±500, 1H NMR) or 125 MHz (Varian Unity ±500, 13C NMR) VRX instruments. ESIMS spectra were recorded on a Waters Xevo G2-S Q-TOF LC mass spectrometer (Waters Corporation, Milford, MA, USA).

3.3.2 Plant material

Root bark of *Diospyros mafiensis* F. White was collected December 23, 2014, at the location S06˚53’33’’ E39˚06’01”, 182 m in Kisarawe, Pugu, Dar es Salaam, Tanzania. A voucher specimen has been deposited in the Botany Department Herbarium, University of Dar es Salaam, Tanzania, for future reference (Voucher No. FMM 3693). The root bark was air-dried in the shade for five days. The dry root bark was milled using a laboratory mill (Model 4, Martha R. Thomas Company, Philadelphia, PA, USA). The milled plant material was shipped to Michigan State University for further analyses.

3.3.3 Fungal strains, growth medium, and growth conditions

Wild-type strains of aflatoxigenic molds *A. flavus* (AF3357), and *A. parasiticus* (SU-1, ATCC56775) and a mutant strain of *A. parasiticus* B62 were used throughout this study. The mutant *A. parasiticus* B62 strain was used for screening the anti-aflatoxigenic activities and aflatoxin reduction efficacies of the methanolic extract and fractions of the plant material. All strains were grown on glucose minimal salts (GMS), which is a chemically defined medium that was prepared as previously described (Tice and Buchanan, 1981). The pH of the medium was adjusted to 4.5 using 1M NaOH. Molds were center-inoculated onto Petri dishes and allowed to grow in the dark in an incubator at 30˚C for 5 days for screening and 10 days for bioassays of isolated bioactives against wild-type strains *A. flavus* AF3357 and *A. parasiticus* SU-1, ATCC56775.

3.3.4 Screening *Diospyros mafiensis* root powders and methanolic extracts using *A. parasiticus* strain B62

A. parasiticus strain B62 accumulates the brightly colored red pigment, norsolorinic acid (NA) (Lee et al. 1971), in the colony and surrounding growth medium (Roze et al. 2011). The disappearance of red coloration following treatment in the growth medium provides visual evidence of aflatoxin biosynthesis inhibition (Figure 3.1). Dry root powders (10 g) were placed in a cell culture dish (150 x 25 mm) and evenly spread at the bottom of the plate. Three small Petri dish (60 x 15 mm) covers were filled with Potato Dextrose Agar (PDA) (10 mL) growth medium. Conidiospores (1x 10^4 CFU/plate) of *A. parasiticus* were center-inoculated onto PDA agar medium solidified in each of small Petri dish covers. The three inoculated Petri dish covers were placed inside a larger dish that contained root powders evenly distributed at the bottom of the dish. Then, the lid of the larger dish was covered and sealed with parafilm to prevent the

escape of root volatiles and the smaller dishes inside the larger dish were open to allow free interactions of gases emanating from the root powders to the growing fungus. The control set was prepared the same way, but the larger dish contained no root powders (Roze at al. 2007; Roze et al. 2011) (Figure 3.1A). The fungus was allowed to grow in the dark at 30˚C for three days.

An appropriate mass of powdered root methanolic extract (25, 50, and 250 mg) (extraction method is described in next section) was dissolved in 1 mL of dimethylsulfoxide (DMSO) to make stock solutions containing 25, 50 and 250 mg/mL, respectively. Flat-bottomed 6-well culture plates (SIAL0516, Sigma-Aldrich, St Louis, MO 63103, USA) were used to grow the molds in triplicate. From each stock concentration, 10 µL were placed into each of the three plates per treatment. Then molten GMS agar tempered to 50˚C (5 mL) was poured into each plate while shaking to ensure homogeneous mixing of the contents. Plates were allowed to cool and solidify the agar. The concentrations in the growth medium were 50, 100, and 500 µg/mL, from the stock solutions 25, 50, 250 mg/mL, respectively. Controls included (1) GMS without extract and (2) GMS without extract but with 10 µL DMSO. Then, conidiospores (1×10^4 CFU/plate) of *A. parasiticus* B62 were center-inoculated onto the GMS agar medium of each plate and incubated in the dark at 30˚C for 5 days.

3.3.5 (a) Bioassay-guided extraction and isolation

The plant material was initially extracted sequentially at room temperature with methanol, ethyl acetate and hexane. Bioassays of resulting extracts showed activity limited to methanolic extract. Subsequently, powdered root barks (200 g) were extracted with methanol (1.5 L, 24 h x3), and evaporation of the solvent under vacuum afforded a powdered extract (57.30 g). An aliquot (20 g) was stirred in methanol (200 mL, 1 h) and centrifuged (relative centrifugal force =

107) at room temperature for 10 min to afford residue **A** (0.75 g, plant material) and supernatant. The supernatant was evaporated under vacuum to obtain methanol-free reddish residue **B** (19.13 g). This residue (19.13 g) was then mixed with hexane (200 mL) and stirred for 1h and centrifuged at room temperature for 10 min to afford precipitate **C** (16.66 g) and supernatant, which was evaporated under vacuum to obtain oily fraction **D** (2 g). The precipitate **C** was mixed with 200 mL of ethylacetate, stirred for 1h, and centrifuged at room temperature for 10 min to afford a precipitate **F** (12.22 g) and supernatant **E**. Evaporation of ethylacetate from the supernatant under vacuum afforded fraction **E** (4.15 g). An aliquot of fraction **E** (350 mg) was mixed with acetone (6 mL) and stirred for 1 h and centrifuged at room temperature for 10 min to obtain precipitate **E1** (17.5 mg) and supernatant. The precipitate **E1** (17.5 mg) was soluble in methanol. To the acetone supernatant, hexane (7 mL) was added and the mixture was stirred for 1h and centrifuged at room temperature for 10 min to obtain subfraction (residue) **E2** (133 mg). The supernatant, acetone-hexane mixture, was evaporated to obtain subfraction **E3** (198 mg) (Alexander-Lindo et al. 2004).

Fractions **A**, **B**, **D**, **E,** and **F**, and sub-fractions **E1**, **E2**, and **E3** were distinct, as indicated by TLC analyses and were screened using *A. parasiticus* B62 grown in the dark at 30˚C for 5 days (see Figure 3.2). The subfraction **E1** was inactive. All fractions (**A**–**F**) and sub-fraction **E2** were weakly active as indicated by Figure 3.1C and Figure 3.2. The subfraction **E3** was the strongest and it was preferentially selected for isolation, purification and characterization of bioactive compounds. An aliquot of **E3** (120 mg) was purified by preparative TLC ($CHCl_3$: MeOH 30:1 v/v, two runs) to yield compounds **1** (23 mg) and **2** (9.2 mg) (Georges et al. 2008; Zhang et al. 2015; Zhang et al. 2016).

3.3.5 (b) Characterization of compounds 1 and 2

Compound 1: Red solid; ^{1}H NMR (500 MHz, $CDCl_3$): 11.85 (1H, s, 5-OH), 11.48 (1H, J = 10.8 Hz, 5'-OH), 7.48 (2H, d, 5.4 Hz, H-8, H-8'), 7.11 (1H, s, H-6), 6.85 (1H, d, 12.7 Hz, H-3), 4.01 (1H, s, H-3'), 3.96 (1H, s, H-2'), 2.44 (3H, s, H-7CH_3), 2.27 (3H, s, H-7'CH_3)(Figure A2); ^{13}C NMR (125 MHz, $CDCl_3$): 195.3, 195.1 (C-4'), 189.6, 189.4 (C-1'), 188.8 (C-4), 182.4 (C-1), 161.4 (C-5), 159.2 (C-5'), 148.7 (C-7), 147.5 (C-7'), 145.5, 145.4 (C-2), 138.9, 138,7 (C-3), 129.1 (C-9, C-9'), 124.2 (C-6'), 121.3 (C-6), 121.1 (C-8, C-8'), 113.1 (C-10'), 112.1 (C-10), 55.4 (C-2'), 55.1 (C-3'), 22.3 (C-11), 22.1 (C-11'). These data revealed that compound **1** was diosquinone (DQ). Based on spectral data, DQ was previously reported from the roots of the same plant (Khan and Rwekika, 1999).

Compound 2: Red solid; ^{1}H NMR (500 MHz, CD_3OD): 7.45 (1H, s, H-8'), 7.36 (1H, s, H-8), 6.92 (1H, H-6), 3.99 (2H, dd, H-2',H-3'), 2.40 (3H, s, H-7CH_3), 2.26 (3H, s, H-7'CH_3) (Figure A3); ^{13}C NMR (125 MHz, CD_3OD): 197.4, (C-4'), 191.7 (C-1'), 190.4 (C-4), 182.1 (C-1), 170.1 (C-3), 163.1 (C-5), 161.8 (C-5'), 150.9 (C-7), 150.1 (C-7'), 136.6 (C-2), 133.6 (C-9, C-6'), 131.3 (C-9'), 121.7 (C-6), 121.5 (C-8'), 120.4 (C-8), 113.2 (C-10'), 113.1 (C-10), 56.8 (C-2'), 56.5 (C-3'), 22.3 (C-11), 22.2 (C-11') (Figure A4). HRESIMS: m/z 405.0621 ($[M-H]^-$ (calcd for $C_{22}H_{13}O_8$, 405.0610) (Figure A5). These data revealed that compound 2 was 3-Hydroxydiosquinone (3HDQ). The molecular ion, $[M-H]^-$, at m/z 405, 16 amu higher than that of DQ, indicated that 3HDQ contained additional oxygen functionality in its structure. This new oxygen functionality assigned as a hydroxyl group at C-3 and resonated upfield at δ 170.1 in its ^{13}C NMR spectrum (Figure A4) was confirmed by the absence of the proton signal at δ 6.85 in its ^{1}H NMR spectrum (Figure A3) when compared to the ^{1}HNMR spectrum of DQ (Figure A2).

3.3.6 Inhibitory effects of DQ and 3HDQ on the vegetative growth of wild-type strains *A. flavus* AF3357 and *A. parasiticus* SU-1, ATCC56775

Diosquinone (DQ) (5 mg) was dissolved in 200 µL of DMSO to make a stock solution with a concentration of 0.025 mg/µL (w/v). Using this stock solution, five serial dilutions were carried out by taking 100 µL of stock solution **I** and mixing it with 100 µL of DMSO. Serial dilutions were conducted to make stock solutions **II**, **III**, **IV**, and **V**. From each stock solution, 10 µL were transferred into the test well plate (in triplicate for each stock solution) and 5 mL of GMS agar was poured into each test well while shaking gently to ensure homogeneous mixing of the contents in order to get concentrations 50, 25, 12.5, 6.25, and 3.125 µg/mL, respectively, as final concentrations in the test well plates. The highest concentration (100 µg/mL) was prepared by transferring 20 µL from the stock solution into 5 mL GMS plate. Thus, dose levels applied for the inhibitory experiments were 100, 50, 25, 12.5, 6.25, and 3.125 µg/mL. The GMS agar medium was left to solidify in the test well plates before inoculation. Serial dilutions of 3HDQ were prepared in the same way that resulted in the same final concentrations of 100, 50, 25, 12.5, 6.25, and 3.125 µg/mL in the test well plates. *A. flavus* (AF3357) and *A. parasiticus* (SU-1, ATCC56775) were exposed to such DQ and 3HDQ by allowing them to grow on the surface of treated GMS growth medium (5 mL) in the test well plates. The plates that contained GMS only or GMS with DMSO only were the positive and negative controls, respectively. Six-well culture plates (SIAL0516, Sigma-Aldrich, St Louis, MO 63103, USA) were used throughout this study. Conidiospores (1×10^4 CFU/plate) of each fungal strain were center-inoculated into each test well and incubated in the dark at 30˚C for 10 days. Fungal growth was estimated by measuring colony diameter in perpendicular directions for each colony every 24h for 10 days. All colony

diameter measurements were recorded as mean ± standard error (SE) as previously described (Roze et al. 2011). The growth inhibition percentages were obtained by the following formula:

Growth Inhibition (%) = (Control–Treatment)/Control x 100 %

3.3.7 Estimation of fungal sporulation

After 10 days of incubation in the dark at 30˚C on GMS medium, conidiospores of *A. flavus* (AF3357) and *A. parasiticus* (SU-1, ATCC56775) were harvested, and spore numbers in (CFU /plate) for each colony were estimated using a hemocytometer as described previously (Roze et al. 2004). Averages of spore numbers (CFU/plate) for each dose concentration were determined.

3.3.8 Extraction and quantification of aflatoxins from growth medium

Total aflatoxins in the growth medium and mycelia were extracted with 5mL chloroform in 50-mL Falcon tubes (Denville Scientific Inc. South Plainfield, NJ07080, USA). Chloroform (5 mL) was added to a 50-mL Falcon tube containing the sample (chunks (@~ 6 x 6 x 5 mm) of solid medium agar from the test well plate). The chunks were vortexed for 5 s and the mixture allowed to rest for 10 min before withdrawing the extract into a 20-mL scintillation vial. This procedure was repeated three times and the extracts were dried completely under a stream of nitrogen gas, and each vial was reconstituted with 500 µL of 70% methanol (Roze et al. 2004). Five µL of the reconstituted solution were dissolved in 1000 µL of 70% methanol, and the solution was vortexed for 30 s to obtain the final sample solution (pH = 6.7). Then, total aflatoxin in the sample was quantified using Veratox® direct competitive enzyme-linked immunosorbent assay (ELISA) as described by the manufacturer (Neogen Corporation, Lansing, MI, USA). Averages of total aflatoxin (ng/plate) at each dose level were determined and recorded.

3.3.9 Statistical analysis

Statistical analysis was conducted using Duncan's method for pairwise comparisons, using SigmaStat one-way analysis of variance (One-Way ANOVA) scientific statistical software, version 11.0 from Jandel Corporation, San Rafael, California, USA.

3.4 Results

3.4.1 Screening *D. mafiensis* root powders and methanolic extracts using *A. parasiticus* B62

The root powders of *D. mafiensis* (R3) decreased vegetative growth and NA production by *A. parasiticus* B62 compared to the untreated control (Figure 3.1A). Crude methanolic extracts of *D. mafiensis* root bark effectively inhibited both vegetative growth and NA production of *A. parasiticus* (Figure 3.1B). Vegetative growth and NA decreased drastically with increase in dose of the extract from 50 to 500 µg/mL as compared to the controls (B62 only and DMSO).

3.4.2 Bioassay-guided extraction and isolation

Except the oily fraction **D** that weakly inhibited vegetative growth and NA production, fractions **A**, **B**, **E**, and **F** weakly inhibited vegetative growth but strongly inhibited NA production by *A. parasiticus* B62 in a dose-dependent manner (Figure 3.2). Fraction **E** exhibited the strongest activity against *A. parasiticus* B62 growth and NA production (Figure 3.2). Thus, fraction **E** was selected for further fractionation to obtain sub-fractions **E1**, **E2**, and **E3**. Sub-fraction **E1** was inactive against the growth of *A. parasiticus* B62. Conversely, sub-fraction **E2** inhibited *A. parasiticus* B62 vegetative growth more weakly than **E3**, but both were strong inhibitors of NA productions. Sub-fraction **E3** was the most potent against the vegetative growth of *A. parasiticus*. From this subfraction, two bioactive compounds were isolated, purified,

characterized, and identified as Diosquinone (DQ) and 3-Hydroxydiosquinone (3HDQ) (Figure 3.1C & 3.1D). The spectral data of DQ were in agreement with Khan and Rwekika (1999) who first isolated and characterized it from *D. mafiensis*. We report here for the first time the spectral data of a new analog of DQ, called 3HDQ.

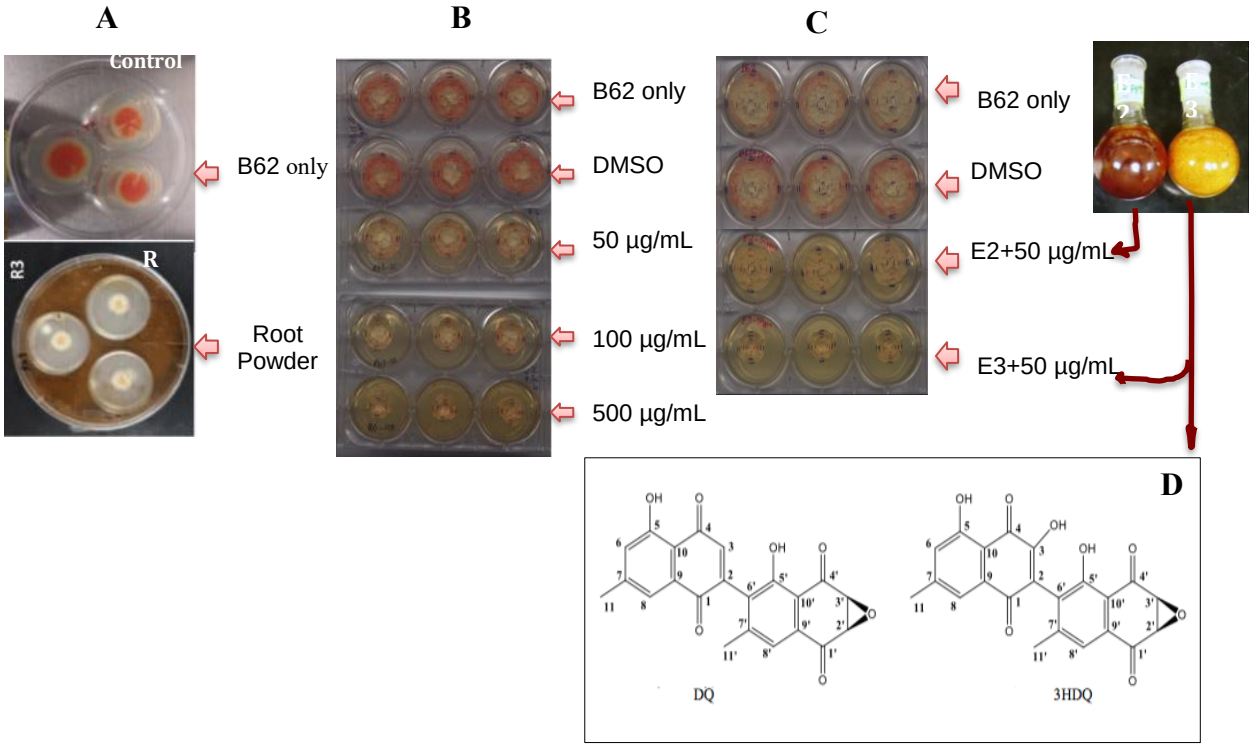

Figure 3.1: Inhibitory effects of root powders of *D. mafiensis* on *A. parasiticus* B62 grown on PDA for 3 days (A); crude methanolic extracts (B); and sub-fractions E2, and E3 (C) obtained from fraction E on vegetative growth and NA production by *A. parasiticus* B62. B62 was grown onto GMS in the dark at 30˚C for five days. The image was obtained on the fifth day of incubation. Controls: B62 only and B62 with DMSO. Decrease in NA intensity indicates aflatoxin reduction due to the plant extract. Purification of E3 using preparative chromatography afforded two biologically active compounds DQ and 3HDQ (D).

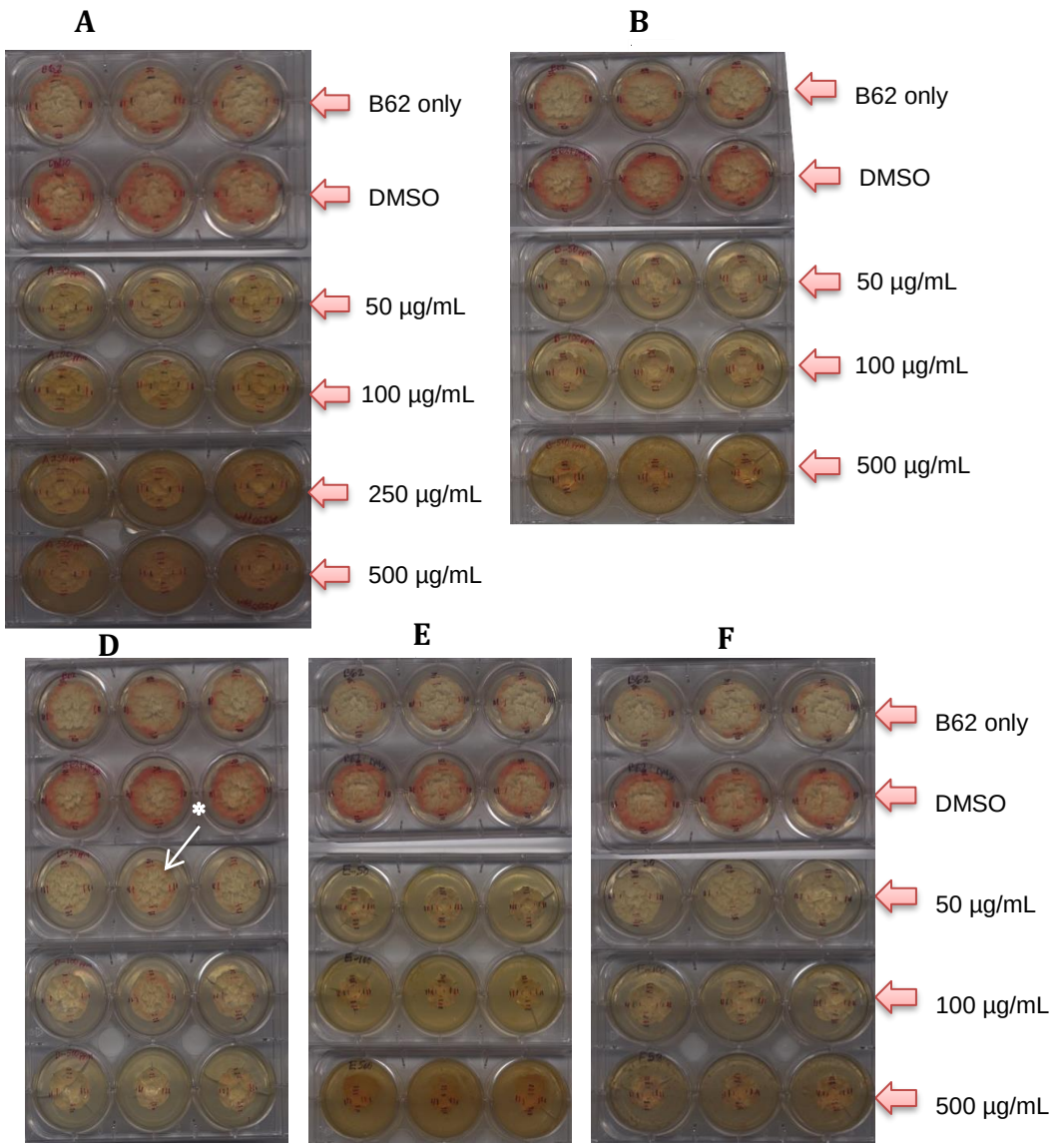

Figure 3.2: Screening the inhibitory effects of fractions A, B, D, E, and F fractionated from crude methanol extract of *D. mafiensis* root bark on vegetative growth and NA production by a mutant *A. parasiticus* B62. B62 was grown onto GMS in the dark at 30˚C for five days. Images were taken on the 5th day of incubation. B62 (grown without extract) and DMSO (B62 were treated with 10µL DMSO) were controls. Decrease in NA intensity indicates aflatoxin reduction ability of the plant extract. *Note that fraction D did not eliminate NA completely. E was the most potent fraction and was selected for fractionation and isolation of the bioactive compounds.

3.4.3 Inhibitory effects of DQ and 3HDQ on the vegetative growth of wild-type strains *A. flavus* AF3357 and *A. parasiticus* SU-1 (ATCC56775)

Figures 3.4A and 3.4B show growth inhibitory effects of DQ and 3HDQ assessed at concentrations ranging from 3.125 to 100 µg/mL on *A. flavus* and *A. parasiticus* grown on GMS for 10 days. At the highest concentration (100 µg/mL), DQ weakly but significantly inhibited ($p < 0.05$) *A. flavus* (43 %) and *A. parasiticus* (34 %) growth compared with the control. There was no change in the level of inhibition of vegetative growth of *A. parasiticus* by DQ from 12.5 to 100 µg/mL. The 50% minimum inhibitory concentration (MIC_{50}) values of DQ in *A. flavus* and *A. parasiticus* were all greater than 100 µg/mL. In contrast, at the highest concentration (100 µg/mL), 3HDQ significantly ($p < 0.05$) inhibited the vegetative growth of *A. flavus* (64%) and *A. parasiticus* (56%). No significant difference in inhibition of vegetative growth of *A. flavus* was observed by 3HDQ from 25 to 100 µg/mL. The MIC_{50} values for 3HDQ were 14.9 µg/mL on *A. flavus* and 39.1 µg/mL on *A. parasiticus* (Figure 3.4A & 3.4B, respectively). Vegetative growth of *A. flavus* was more susceptible to DQ and 3HDQ than *A. parasiticus*. Significantly, 3HDQ was more potent for both *A. flavus* and *A. parasiticus* than DQ especially at doses > 6.25 µg/mL (Figure 3.4A & 3.4B).

Of particular interest, 100 µg/mL of DQ caused a complete loss of green pigmentation in colonies of *A. flavus* whereas a similar dose of 3HDQ did not cause loss of greenish pigmentation in the colonies of the same fungus suggesting that DQ at doses ≥ 100 µg/mL exerts morphological alterations and disrupts ability to form pigments (Figure 3.3). However, we failed to observe this phenomenon in *A. parasiticus* because it did not form greenish pigmentation at all doses (Figure 3.3).

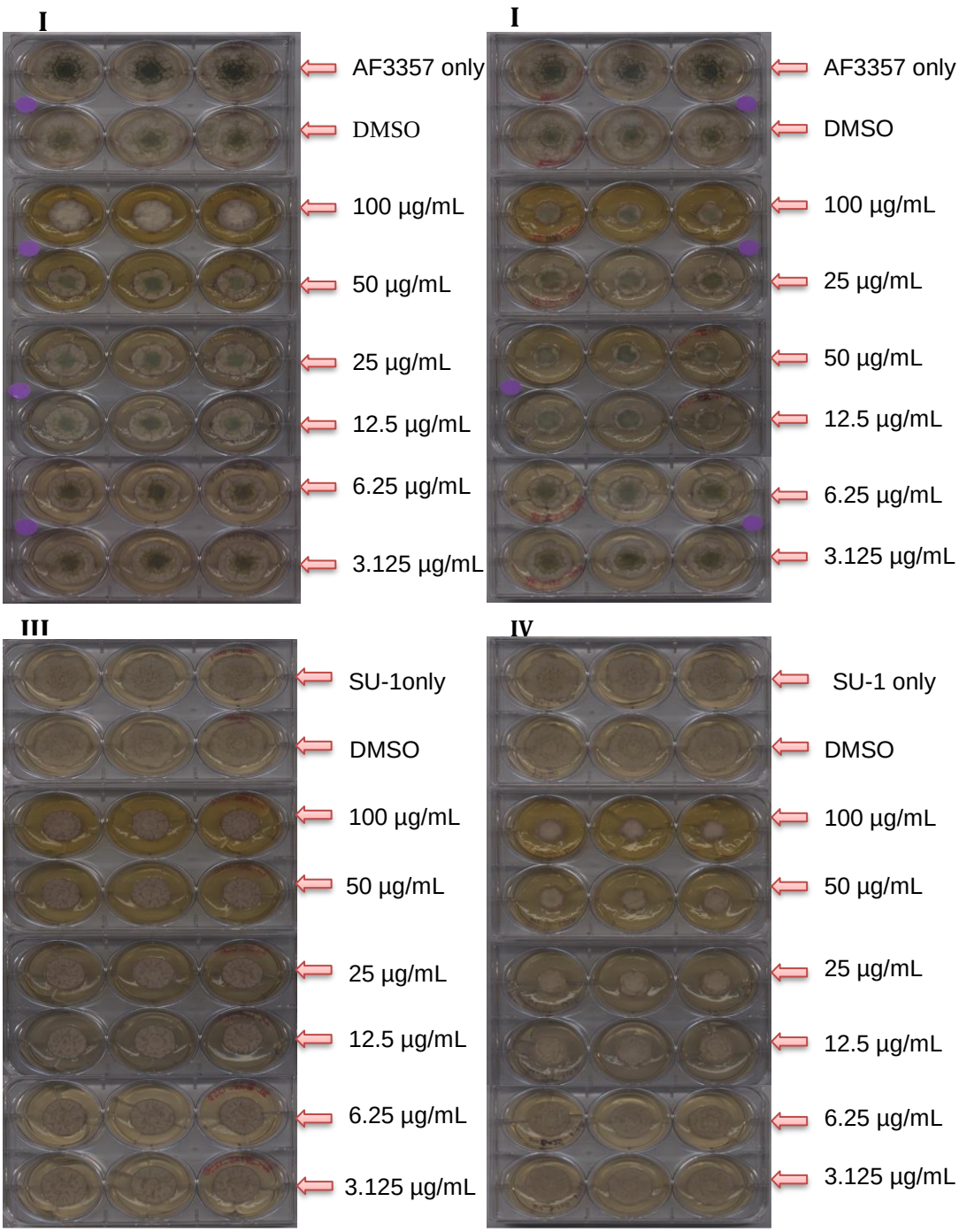

Figure 3.3: Dose-response inhibitory effects of DQ and 3HDQ on vegetative growth of *A. flavus* (I and II, respectively); and on *A. parasiticus* (III and IV, respectively). The spores (1x104 CFU/plate) of the fungus were center-inoculated onto GMS agar and exposed to various doses (3.125 to 100µg/mL) of DQ and 3HDQ for 10 days in the dark at 30˚C. Controls GMS agar plates did not contain the compounds. Positive control GMS agar plates contained the fungus only while the negative control contained 10µL DMSO. Three independent colony diameters were recorded for each concentration as previously described (Roze et al. 2011). At 100µg/mL, DQ caused disappearance of green pigmentation in *A. flavus*. By comparison, 100µg/mL of 3HDQ did not eliminate green pigmentation in *A. flavus*.

3.4.4 Impact of DQ and 3HDQ on fungal sporulation

Conidiospore number for both mold strains decreased significantly ($p < 0.05$) in a dose-dependent manner when they were exposed to increasing doses of DQ and 3HDQ as compared with the controls (Figure 3.4C & 3.4D). For example, 100 µg/mL of 3HDQ strongly decreased conidiospore numbers from 1.7 x 10^6 (control AF3357) to 2.7 x 10^5 spores/ plate (98% reduction of sporulation) in *A. flavus* after 10 days of growth (Figure 3.4C). By comparison, 100 µg/mL of 3HDQ was less effective in decreasing conidiospore number from 2.4 x 10^5 in control SU-1 to 1.2 x 10^5 spores/plate (52% reduction of sporulation) in *A. parasiticus* grown for 10 days (Figure 3.4D). In contrast, DQ was equally potent at reducing conidiospore number in both fungal strains after exposure to 100 µg/mL for 10 days of incubation. DQ reduced conidiospore number from 1.5 x 10^6 in control AF3357 to 3.6 x 10^5 spores/plate (76% reduction of sporulation) in *A. flavus* (Figure 3.4C) exposed to DQ for 10 days and decreased conidiospore number in *A. parasiticus* from 2.6 x 10^5 in control SU-1 to 5.8 x 10^4 spores/plate (77% reduction of sporulation) (Figure 3.4D) after exposure for 10 days. Overall, 3HDQ (with the exception of the 100 µg/mL dose) exhibited lower ability to reduce conidiospore number in both fungi than its counterpart DQ. This suggests that although the DQ was a weaker inhibitor of radial growth (Figure 3.4B), it reduced conidiospore number more effectively.

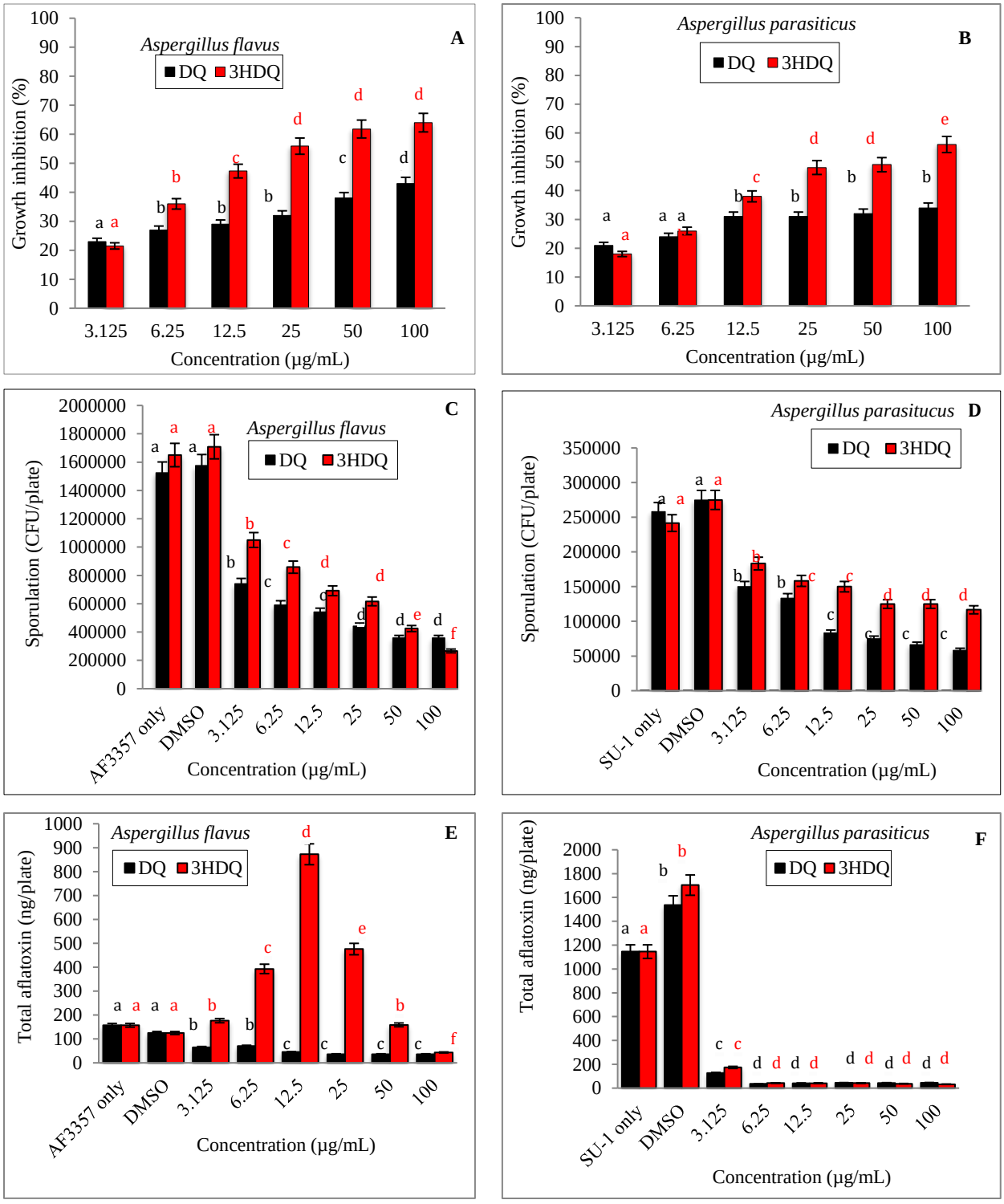

Figure 3.4: Inhibitory effects of DQ and 3HDQ on vegetative growth of *A. flavus* (A) and *A. parasiticus* (B); on sporulation of *A. flavus* (C) and *A. parasiticus* (D); and on total aflatoxin production by *A. flavus* (E) and *A. parasiticus* (F). Differences in data with the same letters are not statistically significant ($p<0.05$) according to Duncan's method of pairwise comparisons test.

3.4.5 Quantification of total aflatoxins extracted from growth medium

After incubation of center-inoculated *A. flavus* and *A. parasiticus* strains in the dark at 30˚C for 10 days, total aflatoxins were extracted from each plate containing growth media. Figures 3.4E and 3.4F show total aflatoxins (ng/plate) presented as mean ± SE of three independent plates for each treatment group against two different wild-type fungal strains. Compared with the control (AF 3357 only), DQ significantly ($p < 0.05$) inhibited aflatoxin production by *A. flavus* at all concentrations after 10 days and total aflatoxin accumulation was inversely proportional to an increase in dose (Figure 3.4E). Higher doses (25 to 100 µg/mL) of DQ inhibited total aflatoxin accumulation by 77.2% (36 *n*g/plate in the treatment group compared with 157 ng/plate in the control, AF3357 only (Figure 3.4E). In contrast, doses consisting of 3.125, 6.25, 12.5, 25, and 50 µg/mL of 3HDQ significantly promoted total aflatoxin production by *A. flavus* to the following amounts 176.5, 393.0, 872.4, 475.9, and 158.8 ng/plate, respectively, compared with 157.1 ng/plate in the control (AF3357 only). Significant inhibition of total aflatoxin production was observed at 100 µg/mL, which caused a 72% reduction of total aflatoxin from 157 ng/plate in the control (AF3357 only) to 43.5 *n*g/plate (Figure 3.4E). The DQ and 3HDQ inhibited total aflatoxin production by *A. parasiticus* at nearly equal efficacy (Figure 3.4F). Compared with 1145.3 ng/plate total aflatoxin in the untreated control (SU-1 only), doses ≥ 6.25 µg/mL of DQ or HDQ nearly eliminated aflatoxin accumulation (44.7 ng/plate, 96.1% aflatoxin reduction; and 32.3 ng/plate, 97.2% aflatoxin reduction, respectively**.**

3.5 Discussion

Exposure of *A. parasiticus* B62 to powdered roots (Figure 3.1A), crude methanolic extracts (Figure 3.1B) and sub-fractions **E2** and **E3** (Fig 3.1C) of *D. mafiensis* inhibited fungal growth and NA-accumulation in the growth medium as compared with the control. Similarly, fractions **A**, **B**, and **F**, exhibited strong NA inhibition (Figure 3.2). The loss of NA accumulation by *A. parasiticus* in initial studies suggested that the chemical constituents in root powders, methanolic extracts and sub-fractions have inherent capabilities to inhibit aflatoxin biosynthesis by interfering with expression of genes and or enzymes responsible for NA production from acetyl-CoA. Early aflatoxin biosynthesis pathway genes including *aflA* (*fas-2*), *aflD* (*fas-1*) and *aflC* (*pksA*) orchestrate the conversion of acetyl-CoA to an unstable polyketide and eventually to NA, the first stable aflatoxin intermediate in the pathway (Yu et al. 2004). The *pksA* gene is located in the aflatoxin biosynthetic pathway gene cluster (1.5 kb) and linked to the *nor-1* gene, which is required for the conversion of NA to averantin (AVN) (Chang et al. 1995). Therefore, disruption of the *pksA* gene prevents NA synthesis in *A. parasiticus* and *A. flavus* (Chang et al. 1995), which could account for the loss of NA in our preliminary work with *A. parasiticus* B62 (Figure 3.1). The purification of sub-fraction **E3** afforded two pure bioactive compounds, DQ and 3HDQ, the latter of which is novel. Although the antibacterial potential of DQ has been reported (Lajubutu et al. 1995), the current study is the first to report on the anti-aflatoxigenic activity of both DQ and 3HDQ against *A. flavus* and *A. parasiticus*.

The inhibitory effects of DQ and 3HDQ on vegetative growth, sporulation and aflatoxin production by wild-type strains of *A. flavus* and *A. parasiticus* are shown in Figure 3.4. Based on these results, it is reasonable to assume that concentrations greater than 100 µg/mL of the two compounds can completely eliminate total aflatoxin accumulation by the molds. Notably, the

ability of 3HDQ to stimulate aflatoxin production sixfold in *A. flavus* at lower concentrations as well as the ability of DQ to strongly inhibit aflatoxin production even at lower concentrations without adversely impacting the vegetative growth of the molds are not entirely surprising. Similar results were reported for an essential oil (consisting of eugenol and acetyleugenol) extracted from *Piper betle*, which promoted aflatoxin production by *A. flavus* as compared to the control at 0.1 µg/mL (Prakash et al. 2010). When the dose was increased to 0.6 µg/mL, complete inhibition of aflatoxin production was observed. The action of 3HDQ against *A. flavus* illustrates that some specific plant-derived compounds are capable of decreasing radial filamentous growth without inhibiting conidiation or aflatoxin production at a lower concentration. On the other hand, the action of DQ is congruent with bioactive compounds known to inhibit aflatoxin production completely without exerting severe effects on the vegetative growth or conidiation (Fajardo et al. 1995). Jayashree and Subramanyam (1999) reported that eugenol significantly inhibited aflatoxin production by *A. parasiticus* without inhibiting vegetative growth. Similarly, Roze and coworkers (2011) reported a more than 90% reduction in aflatoxin accumulation in *A. parasiticus* using volatile compounds from willow bark (*Salix acutifolia*), which concurrently promoted sporulation by 20% as compared to the control. Another study reported a substantial decrease in aflatoxin production by *A. flavus* and *A. parasiticus* exposed to carvacrol and trans-cinnamaldehyde even though these compounds caused minimal growth inhibition on both molds (Yin et al. 2015).

The modes of action of DQ and 3HDQ at the genetic level against these molds are unknown at this point and are subject to further investigation. However, our data demonstrate that the two candidates exert differential impacts on these fungal species. The bioactive DQ and HDQ compounds are highly conjugated phenolic structures, suggesting that they are strong

antioxidants and their inhibitory activity to aflatoxin synthesis may be attributed to such conjugated structures. Previous studies demonstrate that strong oxidizing compounds promote aflatoxin accumulation while strong antioxidants inhibit aflatoxin accumulation (Fanelli and Fabbri 1989; Reverberi et al. 2006). Perhaps related to the oxidizing power, we propose that the hydroxyl group at carbon 3 of 3HDQ may be a critical site for triggering generation of reactive oxygen species (ROS), which are thought to stimulate aflatoxin biosynthesis in *A. flavus*. The stimulation of aflatoxin synthesis may be a defensive response mechanism against ROS to protect the organism from oxidative stress (Grintzalis et al. 2014; Roze et al. 2015). This may explain at least in part why exposure of *A. flavus* cells to lower doses (3.125, 6.25, 12.5, 25, and 50 µg/mL) of 3HDQ caused the fungus to produce more total aflatoxin (872 ng/plate at 12.5 µg/mL) than untreated *A. flavus* (AF3357 only), which produced 157 ng/plate. We speculate that at lower concentrations, the OH at carbon 3 of 3HDQ is amenable to losing an electron from the oxygen atom (can be mediated by ferric (III) ions available in the cytosol) to create radical cations. These free radical cations might serve more as pro-oxidants than antioxidants. Pro-oxidants induce lipid peroxidation creating more ROS, which in turn promote aflatoxin biosynthesis in an attempt to offset the stressful oxidative environment (Grintzalis et al. 2014; Roze et al. 2015). In contrast, DQ did not stimulate aflatoxin production in *A. flavus* (Figure 3.4E) suggesting that the absence of OH at carbon 3 enhances its ROS quenching ability resulting in the molecule serving more as an antioxidant than pro-oxidant. This predicted antioxidant activity makes it a better ROS scavenger. The scavenging strength of antioxidants has been reported to be necessary for inhibiting aflatoxin production (Reverberi et al. 2006). Since DQ inhibited aflatoxin production in each of the fungal strains even at low concentrations without severely impacting growth, it may be a better free radical scavenger than 3HDQ.

Furthermore, DQ's proposed antioxidant activity appears to be more pronounced in *A. parasiticus* than in *A. flavus*. Both bioactive compounds equally inhibit aflatoxin biosynthesis in *A. parasiticus* (Figure 3.4F) suggesting that the presence of OH at C3 in 3HDQ does not guarantee weak antioxidant activity across fungal strains. Furthermore, both DQ and 3HDQ were equally better inhibitors of aflatoxin production against *A. parasiticus* than *A. flavus*. The reason for this difference is not known and it is subject to further investigation but it could be attributed to their genetic differences. Although 3HDQ promoted aflatoxin production in *A. flavus* at lower concentration of 12.5 µg/mL, our data show that its aflatoxin inhibition strength increased steadily from 12.5 to 100 µg/mL (Figure 3.4E). The proposed positive feedback mechanism between antioxidant activity and aflatoxin production inhibition associated with DQ and 3HDQ treatment is in good agreement with observed biological activities of known phenolic antioxidants including butylated hydroxyanisole (BHA) (Fung et al. 1977), cinnamaldehyde (Juglal et al. 2002), and carvacrol (Yin et al. 2015). These studies showed a direct relationship between free radical scavenging power of antioxidants and inhibition of aflatoxin production by *A. flavus* and *A. parasiticus*. In support of this proposed mechanism, phenolic antioxidants are very powerful free radical scavengers in nature and their introduction into the mold cytosol has been reported to decrease free radical levels and aflatoxin production drastically in the cell (Jayashree and Subramanyam 1999).

Most plant-derived inhibitors of aflatoxin biosynthesis have been reported to exert their inhibitory effects at least at one of the following three levels: (1) alteration of the physiological environment of the cell; (2) interference with signal transduction and gene regulatory networks that control the aflatoxin biosynthetic pathway; and (3) blockage of active sites of aflatoxin biosynthesis enzymes (Holmes et al. 2008). In view of level 2 and 3, the quinone moiety in DQ

and 3HDQ provides an alternative mechanistic explanation to account for their ability to inhibit aflatoxin production. We reasoned that the quinone moiety in DQ and 3HDQ mimics the anthraquinone moiety in NA (compare red–highlighted parts of the molecules in Figure 3.5). Thus, we hypothesize that DQ and 3HDQ can competitively bind to the active site in the polyketide synthase (PKS) encoded in the *pksA* gene, which is responsible for the synthesis of NA, the first stable intermediate in the aflatoxin biosynthesis pathway (Figure 3.5A) (Yu et al. 2004). DQ or 3HDQ binding could inhibit the activity of this key enzyme either competitively or via a suicide substrate mechanism thereby down-regulating production of NA and the end products, aflatoxins. This mechanistic hypothesis is consistent with our screening data (Figure 3.1A, 3.1B, and 3.1C) illustrating that exposure of *A. parasiticus* B62 to the root powders, methanolic extract, and fractions **A**, **B**, **F**, **E2** and **E3**, inhibited the accumulation of NA in the growth medium. Furthermore, it is reasonable to speculate that 3HDQ is unable to bind as effectively to the active site of *pksA* in *A. flavus* due to the presence of the OH group at carbon 3 (C3) rendering the compound less able to inhibit *pksA* activity at lower concentrations. However, the binding to *pksA* may be increased at high concentrations in *A. flavus* accounting for the ability to inhibit *pksA* at these higher concentrations (Figure 3.4E). In contrast, this proposed mechanism also suggests that the absence of the OH group at C3 in DQ may enable DQ to fit into the active and bind effectively to the active site of *pksA* in either of the aflatoxin-producing fungi in this study.

The double bond present at carbons 8, 9 of the furofuran (*bis*furan) moiety in aflatoxin B_1 is bioactivated by cellular cytochrome P450 enzymes primarily in liver microsomes of animals generating a highly reactive aflatoxin–8,9–epoxide that accounts for aflatoxin being designated a group 1 carcinogen (Iyer et al. 1994). The carcinogenicity arises from aggressive reactions of the

aflatoxin–8,9–epoxide species with DNA (Iyer et al. 1994) and these adducts block ability of the DNA to be expressed or replicated. It is possible that DQ and HDQ mimic this biological activity of AFB_1 accounting for their capacity to inhibit fungal growth and perhaps conidiospore development as well. Aspergilli compartmentalize aflatoxins in specialized vesicles called aflatoxisomes located in the cytosol enabling them to avoid aflatoxin's deleterious effects on their DNA and protein molecules and to transport them to the cell exterior most likely via exocytosis (Chanda et al. 2009; Roze et al. 2011). Since DQ and 3HDQ are xenobiotic, aspergilli may be unable to compartmentalize them upon introduction into the cell. Thus, the potentially highly reactive 2',3'–epoxide groups of DQ and 3HDQ may be free to intercalate and react with cellular macromolecules such as DNA, and proteins (Figure 3.5B) to form DNA and proteins adducts (da Cruz Cabral et al. 2013). Also, the phenolic nucleus and epoxide groups might damage cell membranes and cause leakage of intracellular macromolecules such as ATP to the cell exterior leading to energy dissipation and cell death (Fung et al. 1977). The OH groups can form hydrogen bonds with the active sites of various enzymes in the cell and disrupt their activity (Farag et al. 1989). Together, these actions are vital to inhibition of vegetative fungal growth, conidiospore development, and aflatoxin biosynthesis.

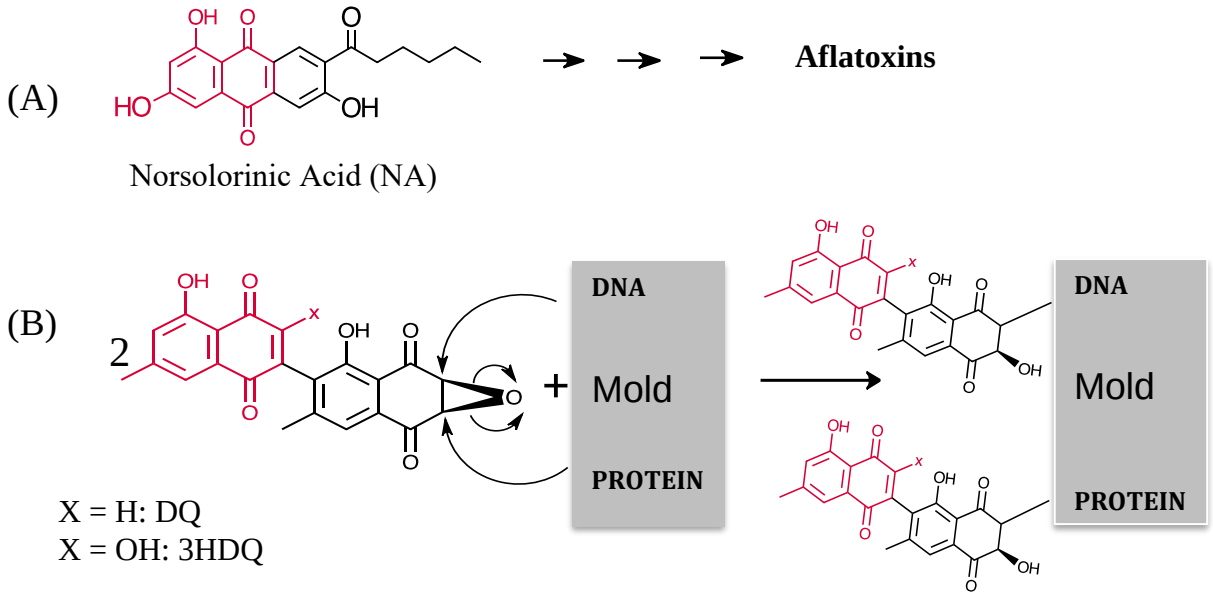

Figure 3.5: The anthraquinone moiety of NA (red-highlighted) might be mimicking the quinone moiety of DQ and 3HDQ in their mode of actions (A). Proposed possible interactions of DQ and 3HDQ with DNA and protein within the cell of the mold (B).

The significance of these findings may be twofold. First, if the "mimicry" theory is correct, then the inhibitory actions of DQ and 3HDQ are most likely specific to *pksA*, a key enzyme in the early stages of aflatoxin biosynthesis. Studying and understanding the structures of inhibitors that can best inhibit/block the activity of this enzyme are crucial in incapacitating the molds to produce aflatoxins (Holmes et al. 2008). The fact that DQ demonstrated stronger inhibition of aflatoxin production (>97 %) without severely impacting the vegetative growth may endorse it as a preferred inhibitor candidate for future studies designed to investigate potential mechanisms by which it blocks *pksA*. Also, the fact that lower doses of 3HDQ stimulated aflatoxin production in *A. flavus* and not *A. parasiticus* may provide an incentive to study how specifically *pksA* might be impacted by binding to 3HDQ. Studies on the chemical structures that enhance or prevent specific binding to *pksA* would be useful in the design of inhibitors of fungal growth and aflatoxin production for the complete elimination of aflatoxin in food and feed in the future.

The long-term goal of this research was to search for inexpensive plant-derived fungicides, which could be used to eliminate or minimize aflatoxin accumulation on economically important crops like corn, peanuts, and tree nuts under storage conditions. The capacity of crude root powders, extracts, and compounds to inhibit vegetative growth as well as NA accumulation (Figure 3.1A) supports that economically challenged farmers in tropics to may use chips of *D. mafiensis* root bark to protect their crops under storage conditions. Therefore, the use of this plant for aflatoxin mitigation would invite the necessity of its domestication and cultivation for large-scale application in tropical countries where it thrives.

3.6 Conclusions

To our knowledge, the work described above represents the first study to report on the antiaflatoxigenic activity of DQ and 3HDQ from *D. mafiensis*. Both compounds were strong inhibitors of aflatoxin production by *A. flavus* and *A. parasiticus*. Thus, they could be used to prevent aflatoxin accumulation in stored food crops. Because *D. mafiensis* has been utilized effectively as a medicinal plant throughout recent history demonstrates promise for the safe application of root powders and extracts to prevent aflatoxin biosynthesis in a practical and safe manner. Toward this end, future work will evaluate the performance of root chips, DQ, and 3HDQ to avoid fungal spoilage and aflatoxin accumulation in food crops and feed under storage conditions. Also, future evaluation of the performance of *D. mafiensis* should consider the versatility of plant materials in relation to seasonality. Most importantly, the toxicity studies of these antifungal natural chemicals are recommended before approved for use in food and feed.

CHAPTER 4: CONCLUSIONS AND RECOMMENDATIONS

In Tanzania, aflatoxin surveillance in oilseeds, cakes and crude oils such as those of sunflower have not received a strict consideration as compared to the staples maize, peanuts, millet and cassava. Sunflower crop is often overlooked and understudied because it is assumed to be less susceptible to aflatoxin contamination. Our results presented in Chapter 3 have clearly proved this perception wrong. They demonstrate that sunflower seeds, and of course, sunflower cakes and crude oils, are not immune to aflatoxin contamination. In our extensive two-year survey conducted in 2014 and 2015 across Tanzania from micro-scale sunflower oil processors in selected towns of Mbeya, Iringa, Morogoro, Dodoma, Singida, Babati-Manyara, and Karatu-Arusha, revealed aflatoxin contaminations in sunflower seeds, cakes, and sediments. The high aflatoxin levels found in sunflower samples from these processors in central Tanzania and its contiguous areas (Morogoro, Dodoma, Singida, and Babati-Manyara) were of particular concern compared with the levels in samples from other regions studied. The extreme aflatoxin levels observed in seeds and cakes from this area prompted us to further determine levels of total aflatoxin from the sediments of their crude sunflower oils. However, none of the crude sunflower oil sediments in the selected areas showed aflatoxin levels of concern except sediments collected from Morogoro sunflower oil processors in 2015 that had shown levels of concerns (42 and 85 ng/g). These crude oil sediment results in Morogoro in 2015 may serve as a wake-up call that when seeds are extremely contaminated with aflatoxin, the cakes, and the crude oils are also likely to be contaminated. They are a powerful reminder that the sediments in the crude oils may be potential carriers of aflatoxin.

Our risk assessment has highlighted that the dietary exposures via sunflower seeds from Dodoma, Morogoro, Singida and Manyara are of a health concern. It alerts that sunflower seeds from this region may harbor aflatoxins, which may also find their way to crude sunflower oils and sunflower cakes. The aflatoxin contamination levels of sunflower seeds in the 2014 and 2015 sunflower season, a 70-kg adult who consumes an average of 36 g of sunflower seeds from Dodoma, Manyara and Morogoro daily is likely to be at risk for aflatoxin-induced liver cancer of approximately one individual per 100,000 per year (four persons per 410,956 per year in Dodoma in 2014, and three persons per 315,866 per year in Morogoro in 2015). The crude sunflower oil sediments showed lower dietary exposures and suggested no significant risk of primary aflatoxin-induced liver cancer in all locations.

The aflatoxin levels and the dietary exposure assessment and population risk for primary cancer risk estimations of this study may be sufficient to raise food and feed safety concerns. Thus, it is our expectation that these data may alert and encourage micro-scale oil processors, the local food authorities such as Tanzania Food and Drug Authority (TFDA) and Tanzania Bureau of Standards (TBS) to take intervention actions for animal and public health protection.

Future studies should perform an extensive sampling to reach the innermost/interiorly placed sunflower seed and cake sacks that we could not reach. Based on the poorly ventilated and damp stores we visited, we believe that these sacks could have even higher levels of aflatoxin than peripherally located sacks that we sampled. In addition, the number of samples per region should be increased and aflatoxin surveys should be performed over a long period of time to be able to draw a more reliable conclusion on the aflatoxin incidence in sunflower zone.

Due to high detection sensitivity, we recommend the use of LC-MS or HPLC methods over ELISA in future for most sensitive aflatoxin analysis. Additionally, LC-MS or HPLC can

analyze concentrations of individual types of aflatoxin, and this information can also be used to identify the distribution of fungal strains in Central Tanzania. Then, conducting biomarker-based dietary exposure assessment and cancer risk characterization is highly recommended.

The poor storage conditions and drought might be the two most important risk factors for central Tanzania and its contiguous areas. Because this region revealed such high levels of total aflatoxin in stored seeds and cakes, perhaps education of micro-scale sunflower farmers and processors on the proper post-harvest handling of the seeds and cakes could be helpful to minimize the aflatoxin levels in food and feed. Furthermore, national forestation and re-forestation programs in central Tanzania and contiguous regions could also help to intervene severe aflatoxin contamination associated with drought.

In an attempt to search for inexpensive and biodegradable natural antiaflatoxigenic chemicals from medicinal plants, *D. mafiensis* (F. White) root powders, root methanolic extracts have shown considerable promise. The results presented in Chapter 4 clearly demonstrate that *D. mafiensis* root powder, and methanolic root extracts are capable of inhibiting the inherent ability of *A. parasiticus* B62 to produce norsolorinic acid. The crude methanolic extract of *D. mafiensis* root powders contained bioactive compounds with varying potencies against *A. flavus* and *A. parasiticus*. Although at lower concentrations (<25 µg/mL), 3HDQ promoted aflatoxin production by *A. flavus*, at the concentration greater than or equal to 100 µg/mL it prevented aflatoxin production by *A. flavus* for over 70%. Interestingly, both DQ and 3HDQ were able to inhibit aflatoxin production by *A. parasiticus* by more than 97% at a lower concentration of 6.25 µg/mL. Because *A. parasiticus* is a great producer of all types of aflatoxins, this severe impact of DQ and 3HDQ on aflatoxin production by this fungal strain suggests that such natural chemical compounds may be useful to control aflatoxin accumulation in food and feed during storage.

However, we do not know the underlying mechanism of action of these candidates, and this creates a knowledge gap to carry out future mechanistic studies. It may be interesting to investigate their mode of action by using PCR technology to identify the target gene (s) within the aflatoxin biosynthetic pathway. Structure-activity relationship and molecular simulation studies could be established using these compounds to find the best inhibitor of aflatoxin biosynthesis pathway. A question like "What would happen to the activity of the 3HDQ if its OH group at carbon three was replaced with a halogen atom such as chlorine, fluorine, and bromine?" would be interesting to find an answer to.

The ultimate application of DQ and 3HDQ would be incorporation into seed storage bags to prevent excessive mold growth under storage conditions. The fact that *D. mafiensis* root powders decreased both growth and NA production suggests that peasants could incorporate root chips of this medicinal plant into the stored seeds to prevent aflatoxin accumulation under storage conditions. Although the rural farmers have been using this plant as a source of traditional medicines (e.g. infusions, decoctions, and topical solutions) throughout recent history, toxicity studies of these compounds and extracts must be performed to confirm their safety before they can find their applicability in food and feed under storage conditions.

APPENDIX

APPENDIX

Table A1: Weights of sediments extracted from sunflower crude oils collected in 2014.

Location/Town	Number of samples	†Weight of sunflower crude oils collected from oil mills **A–G** before extraction of sediments (g)							††Weight of sediments from sunflower crude oils collected from oil mills **A–G** (g)						
		A	**B**	**C**	**D**	**E**	**F**	**G**	**A**	**B**	**C**	**D**	**E**	**F**	**G**
Babati-Manyara	6	45.0	95.1	90.2	94.8	48.2	48.2	n.a.	2.6	8.0	9.9	6.1	3.0	3.3	n.a.
Singida	6	99.2	93.2	87.2	87.1	81.9	86.1	n.a.	8.9	7.2	6.1	5.1	3.3	7.3	n.a.
Dodoma	7	96.4	96.9	95.0	99.3	93.6	95.3	45.9	7.3	7.2	3.5	5.3	6.2	4.7	1.6
Morogoro	4	92.4	26.7	26.9	26.7	n.a.	n.a.	n.a.	8.2	1.9	2.0	1.9	n.a.	n.a.	n.a.

n.a.= not available. †A total weight of sunflower crude oils for all locations was 1751.4 g. †† A total weight of sediments extracted for all locations was 120.7g. Hence, these data indicate that every 14.5 g of sunflower crude oil yielded 1g of sediments. n.a. (not available).

Table A2: Weights of sediments extracted from sunflower crude oils collected in 2015.

Location/Town	Number of samples	†Weight of sunflower crude oils collected from oil mills **A–G** before extraction of sediments (g)							††Weight of sediments from sunflower crude oils from oil mills **A–G** (g)						
		A	**B**	**C**	**D**	**E**	**F**	**G**	**A**	**B**	**C**	**D**	**E**	**F**	**G**
Babati-Manyara	7	22.3	25.8	25.7	22.9	26.0	19.5	21.7	1.1	0.8	1.2	1.5	2.8	0.9	0.9
Singida	7	23.4	19.6	21.9	21.3	25.1	23.8	25.1	1.1	1.4	1.3	1.5	1.2	2.0	2.4
Dodoma	7	21.5	21.7	22.0	26.2	26.0	20.8	22.2	1.2	1.4	1.4	2.7	1.2	1.7	1.3
Morogoro	6	40.6	11.3	21.9	23.3	30.9	23.3	n.a.	3.6	0.7	1.2	0.9	1.2	0.9	n.a.

n.a.= not available. †A total weight of sunflower crude oils for all locations was 635.7 g. †† A total weight of sediments extracted for all locations was 39.2 g. Hence, these data indicate that every 16 g of sunflower crude oil yielded 1g of sediments.

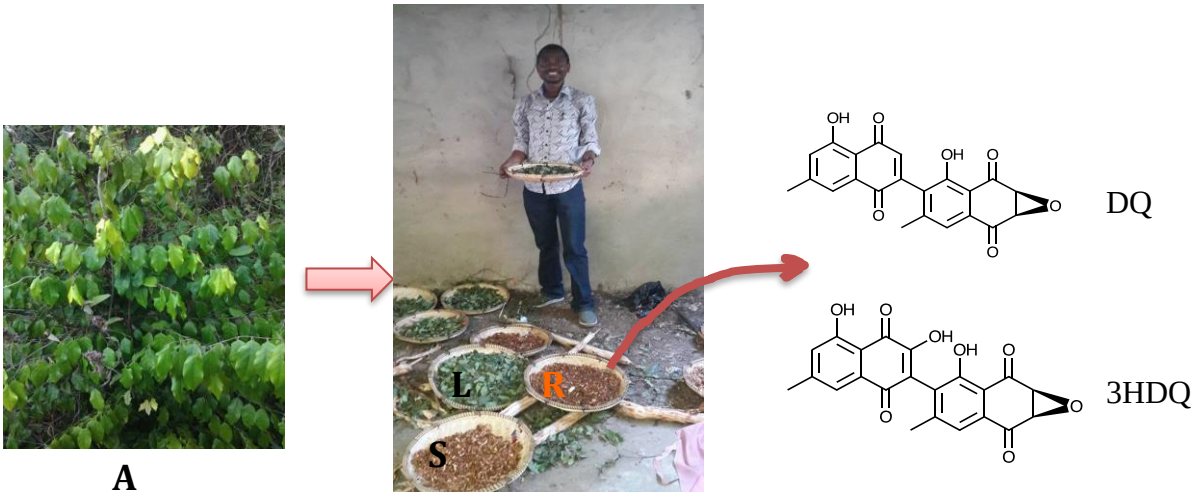

Figure A1: Graphical Abstract: Medicinal plant (*D. mafiensis*) (A); Dried Leaves (L), Stem Chips (S), and Root Chips (R) and isolated bioactive compounds (DQ and 3HDQ) from root (R).

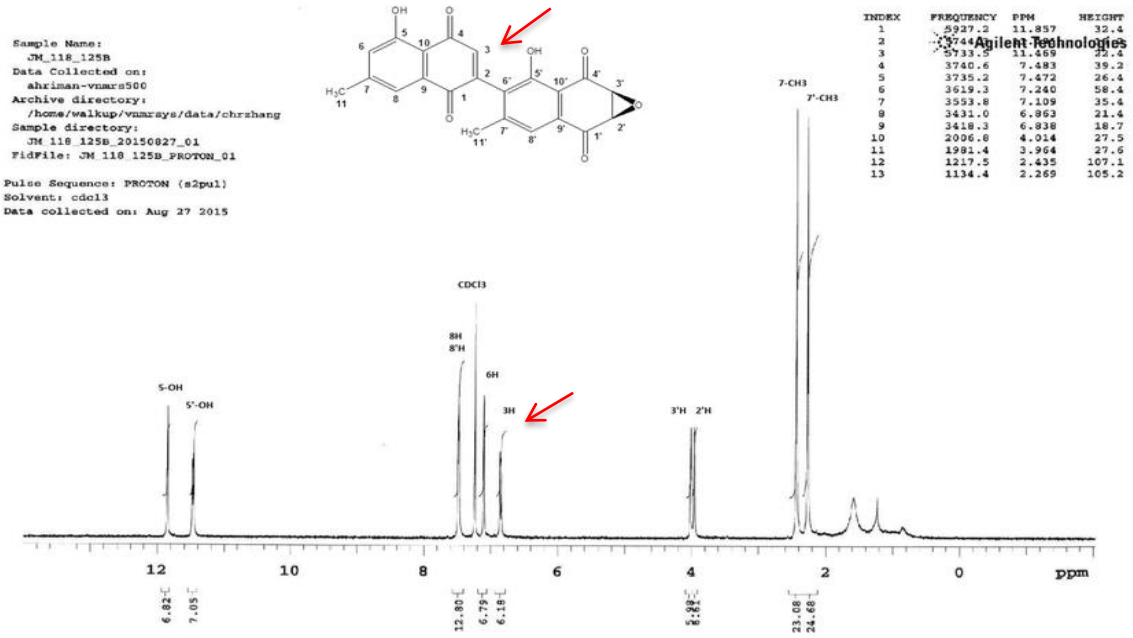

Figure A2: ^{1}H NMR spectrum of DQ. The doublet at δ 6.18 ppm (red-arrowed) corresponds to a proton at carbon 3 position (red-arrowed). This was a diagnostic signal to identify its analogue 3HDQ in Figure 3.1D.

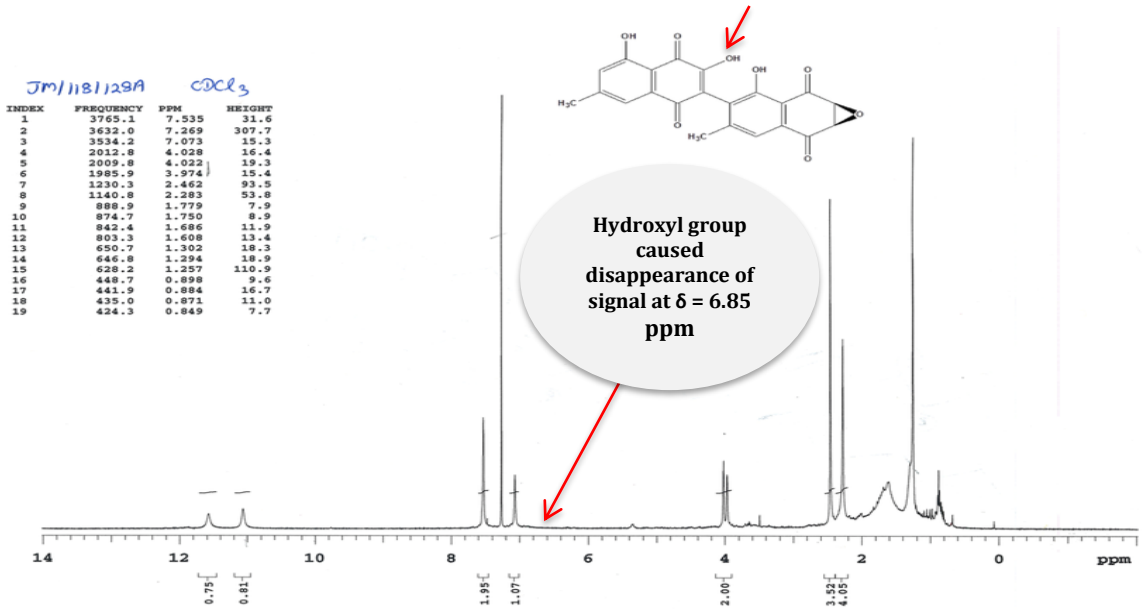

Figure A3: ^{1}H NMR spectrum of 3HDQ. The doublet at δ 6.85 ppm (red-arrowed) corresponds to a proton at carbon 3 position (red-arrowed) disappeared due to hydroxyl group. Compare it with Figure A2.

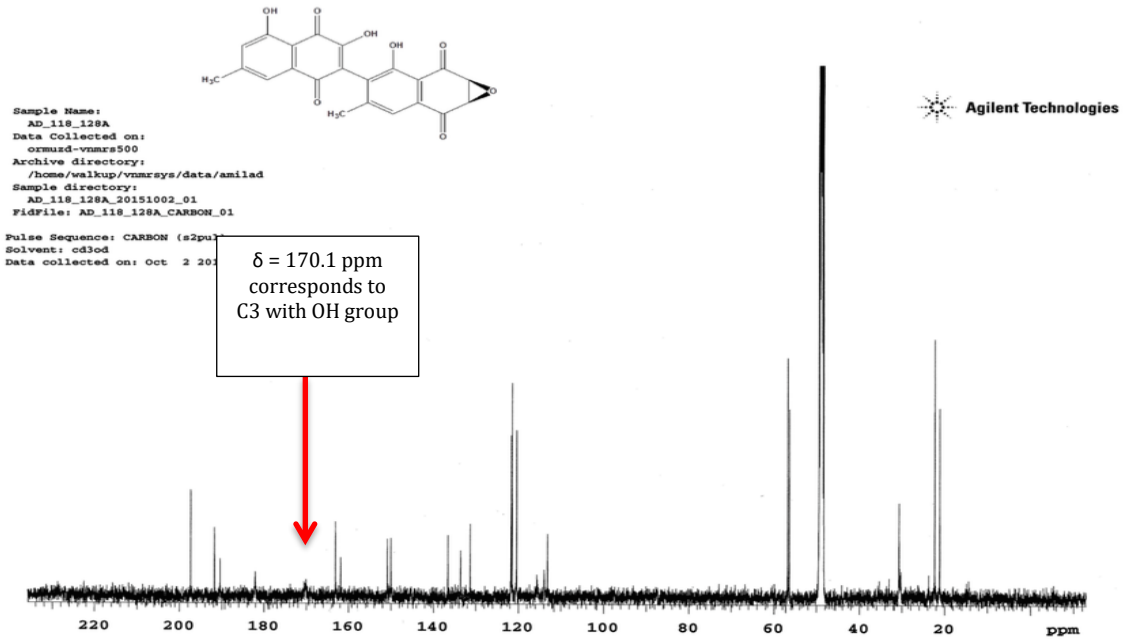

Figure A4: ^{13}CNMR Spectrum of 3HDQ. It confirmed the presence of hydroxyl group at carbon 3 position in 3HDQ.

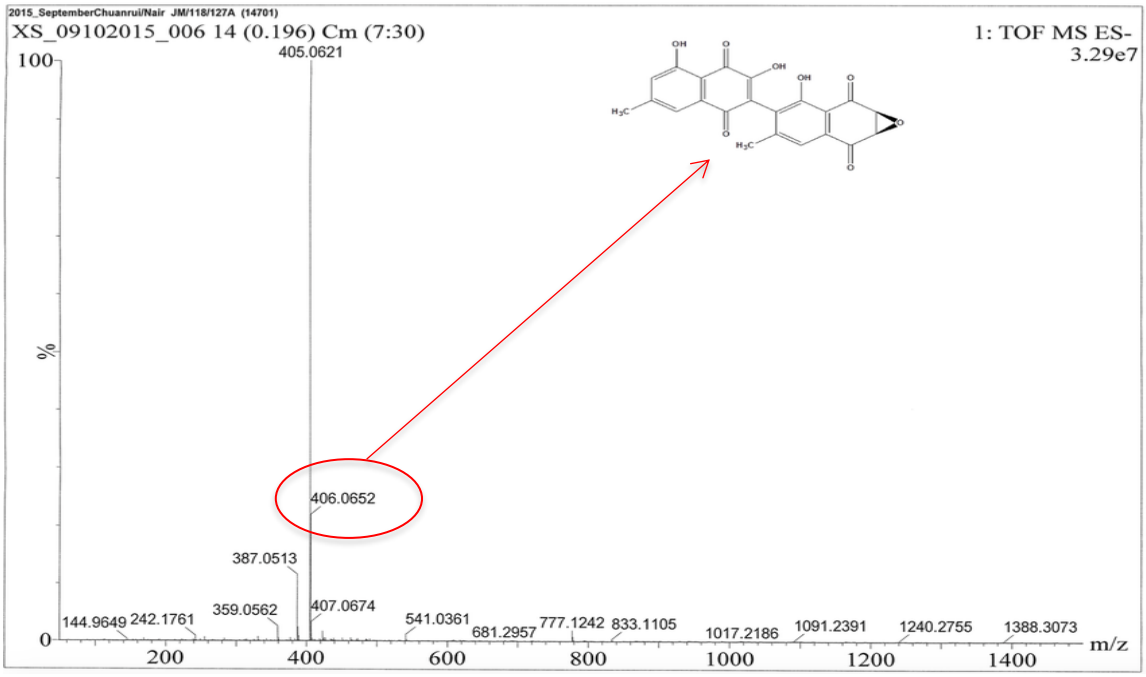

Figure A5: Mass spectrum of 3HDQ. The mass spectrum further confirmed the presence of hydroxyl group at carbon 3 position. The difference in molar masses of the two analogs (DQ from 3HDQ) is 16 a.m.u, which suggests additional oxygen functionality to DQ at carbon 3 position to obtain 3HDQ.

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