Investigation of fungal dissemination by Housefly (*Musca domestica* L.) and contamination of food commodities in selected rural areas in South Africa

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By

JUDITH ZANELE PHOKU

Supervisor: Professor MF Dutton _____

Co-Supervisors: Prof N Potgieter _____

Dr TG Barnard _____

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DECLARATION

The experimental work described in this thesis was carried out in the Food, Environment and Health Research Group, Faculty of Health Sciences, Department of Biomedical Technology, under the supervision of Professor M.F. Dutton, Prof N. Potgieter in the Department of Microbiology, Environmental Health, Domestic Hygiene and Microbial Pathogens Research Group, University of Venda and Dr T.G. Barnard, Water and Health Research Centre, Faculty of Health Sciences, Department of Biomedical Technology, University of Johannesburg, from January 2011 to December 2013. These studies represent my original work and have not been submitted in any form to another University. Where the work of others was used, it has been duly acknowledged in the text.

Signature: _____

JUDITH ZANELE PHOKU

DEDICATIONS

To God all mighty, the creator of the heaven and earth, thank you so much for the mind, courage and strength you have blessed me with to be able to carry out this study.

To my beloved grandparents, I sincerely dedicate this thesis to my late great grandmother Crestinah Silinda, grandparents Mrs Orah Sibusise Siwela (1939-2011) and Mr Siphiwe Daniel Siwela (1937-2011). Indeed I shall forever be grateful for the time I spent with you while on earth and in our hearts you will remain. No last word, no last greeting but in heaven we shall meet again.

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ABSTRACT

Houseflies, *Musca domestica* Linnaeus, are commonest insects of both farms and homes. This species have increasingly overcrowded human dwellings, particularly in rural areas and constitute a health hazard. These flies are not only a nuisance, but they can also transport disease-causing organisms. This may lead to the spread of diseases and also mycotoxin-producing fungi. As a result, frequent exposure to the activity of houseflies will have an impact on public health. Houseflies are dominant in exposed items such as drinking water, food and human faeces. The study was conducted to determine the potential of houseflies in enhancing fungal contamination of food commodities. A total of 183 samples of houseflies (122), maize (15), porridge (19) and water (27) samples collected from Gauteng Province of South Africa were screened for fungi contamination by serial dilution technique on agar plates following DNA sequencing. In this case, 729 fungal isolates were identified with the most commonly isolated fungi belonging to the genera of *Aspergillus*, *Fusarium* and *Penicillium*. On the other hand, *Aspergillus flavus* was by far the most common, followed by *A. niger*, *A. parasiticus* and *A. fumigatus*. The most common *Fusarium* species were *F. verticillioides*, *F. proliferatum*, *F. oxysporum* and *F. culmorum*. Among the *Penicillium* species, *Penicillium aurantiogriseum*, *P. brevicompactum*, *P. oslonii* and *P. verrucosum* were the most common fungi. The incidence rates of fungal contamination per total fungal count isolated in houseflies, porridge, maize and water were recorded with fungal load of 2 x 10^8 CFU/ml, 2x 10^7 CFU/g, 1x 10^7 CFU/g and 1 x 10^2 CFU/g. *Aspergillus*, *Fusarium* and *Penicillium* species recovered were further confirmed and quantified for the production of aflatoxins (AFB_1, AFB_2, AFG_1 and AFG_2), deoxynivalenol (DON), fumonisin B_1 (FB_1) ochratoxin A (OTA) and zearalenone (ZEA) by thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC). The analysis of houseflies isolates by TLC revealed that FB_1 was found with the highest incidence rate of 62 (73%) while, ZEA and DON had the lowest incidence rate of 15 (18%) and 8 (9%). For *Fusarium* isolates (66) from maize, only 11 (17%) of the extracts were positive for FB_1 from *F. verticillioides*. The fluorescence of AFs and OTA viewed under ultra violet light revealed that some isolates were positive showing a light blue fluorescence for AFB_1 and AFB_2, light green fluorescence for AFG_1 and AFG_2, while a blue-green fluorescence was observed for OTA. According to the results on HPLC, *Aspergillus flavus* and *A. parasiticus* were found to be the main producers of AFB_1, AFB_2, AFG_1 and AFG_2, while *A. carbonarius*, *A. niger* and *A.*

ochraceus produced OTA. They produced AFB_1, AFB_2, AFG_1 and AFG_2, either singly or combined. Production of OTA was observed among the species of *P. verrucosum*. Fumonisin B_1 was the most common *Fusarium* toxin produced by mainly *F. verticillioides* and *F. proliferatum*. Co-production of DON, FB_1 and ZEA toxins was observed amongst the *Fusarium* isolates from houseflies and maize. To evaluate the effects of *Fusarium, Aspergillus* and *Penicillium* extracts, *In vitro* experiments were conducted using methylthiozol tetrazolium assay (MTT) on human mononuclear cells. Results revealed that all mycotoxin containing extracts decreased the viability of cells when exposed on mononuclear cells at varying volumes 20, 40 and 80 µl over 24, 48 and 72hrs incubation period. Faecal materials (200) were also collected and analysed for *Fusarium* species using a serial dilution method, while FB_1 was further analysed and quantified by reversed-phase TLC and HPLC. Results revealed the presence of 11 different *Fusarium* species of which *F. verticillioides*, *F. proliferatum*, and *F. oxysporum* were the dominant species. Fumonisin B_1 was recorded at an incidence rate of 65% on TLC while, HPLC results showed that 84% were positive at different ranges of concentration for FB_1. This study showed how houseflies disseminates and contaminates foods and feeds with fungi which produce mycotoxins. The study proved that many of the isolates had the capacity to produce the major mycotoxins (AFBs, DON, FB_1, OTA and ZEA). The extracts of the isolates contained the major mycotoxins and in most cases in combination, and demonstrated to reduce the viability of human mononuclear cells. The study also showed FB_1 presence in faeces is a suitable biomarker for determination of FB_1 exposure. The work conclusively attempted to show that the use of bio-wipe kit in collection of faeces is a rapid and cost effective technique for fumonisins analysis.

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LIST OF ABBREVIATIONS

AA	Acetic acid
ACN	Acetonitrile
AFs	Aflatoxins
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
AFM_2	Aflatoxin M_2
ANOVA	One-way analysis of variance
ATA	Alimentary toxic aleukia
A_w	Water activity
BEN	Balkan endemic nephropathy
BWA	Butanol-water-acetic acid
CAST	Council for Agricultural Science and Technology
CCM	Complete culture media
CDC	Centres for Disease Control and Prevention
CIT	Citrinin
CFU/g	Colony forming units/gram
CFU/ml	Colony forming units/millilitre

CPA	Cyclopiazonic acid
CYA	Czpek yeast agar
DAS	Diacetoxyscirpenol
DCM	Dichloromethane
DEI	Dichloromethane-ethyl acetate-propan-2-ol
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic Acid
DON	Deoxynivalenol
EAs	Ergot alkaloids
EC	European Commission
EFSA	European Food Safety Authority
EHC	Environmental Health Criteria
ELEM	Equine encephalomalacia
ELISA	Enzyme-Linked Immunosorbent Assay
EMAN	European mycotoxin awareness network
EU	European union
FAD	Food and Agriculture Administration
FBs	Fumonisins
FB_1	Fumonisin B_1
FB_2	Fumonisin B_2
FB_3	Fumonisin B_3

FCS	Foetal calf serum
FEHR	Food Environment and Health Research
FHB	Fusarium head blight
FUS X	Fusarenon X
GC/MS	Gas chromatography/mass spectrometry
GIT	Gastro-intestinal tract
GHS	Glutathione
HEK	Human embryonic kidney cells
HIV	Human immunodeficiency virus
HL_{60}	Human leukaemia
HPBLs	Human peripheral blood lymphocytes
HPLC	High performance liquid chromatography
Hrs	Hours
IAC	Immuno-affinity column
IARC	International Agency for Research on Cancer.
ICC	Idiopathic congestive cardiopathy
IPCS	International Program on Chemical Safety
JECFA	Joint FAO/WHO Expert Committee on Food Additives
LC/MS	Liquid chromatography mass spectrometer
LD_{so}	Lethal dose
LOD	Limit of detection

MEA	Malt extract agar
MON	Moniliformin
MTT	Methyl thiazol tetrazolium assay
NIV	Nivalenol
NTDs	Neural tube defects
O_2	Oxygen
OAESA	Ohio Agricultural Experimental Station Agar
OPA	o-Phthaldialdehyde
OTs	Ochratoxins
OTA	Ochratoxin A
OTB	Ochratoxin B
OTC	Ochratoxin C
PCA	Penicillic acid
PAT	Patulin
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
PHA	Phyto-haemagglutinin
ppb	Parts per billion
ppm	Parts per million
PMTDI	Provisional maximum tolerable daily intake

R_F	Retardation factor
RNA	Ribonucleic Acid
ROS	reactive oxygen species
RQC	Roquefortine C
SCF	Scientific Committee on Food
SPE	Strong phase extraction
Spp.	Species
STC	Sterigmatocystin
TDI	Tolerable daily intake
THs	Trichothecenes
TLC	Thin layer chromatography
TEF	Toluene-ethyl acetate-formic acid
TEF 1-α	Translocation elongation factor 1-α
UBB	Ultra biomass bait
UHPLC	Ultra-high performance liquid chromatography
USA	United States of America
USFDA	Unite States Food and Drug Administration
UV	Ultraviolet
WHO	World Health Organization
YES	Yeast extract sucrose agar
ZEA	Zearalenone

LIST OF UNITS

%	Percent
˚C	Degree Celsius
<	Less than
>	Greater than
≥	Greater than or Equal to
±	Plus-minus
µg/ml	Microgram/millilitre
µg/kg	Microgram/kilogram
µl	Microlitre
µm	Micrometer
µM	Micromolar
cm	Centimeter
g	Gram
Hrs	Hours
kg	Kilogram
k	Kilometre
l	Litre
M	Moles
Mins	Minutes
mm	Millimetre

mg/kg	Milligram/kilogram
ng/kg	Nanogram/kilogram
nm	Nanometre
ppb	Parts per billion
ppm	Parts per million
Secs	Seconds
v	Volume

CHAPTER ONE

1.1 GENERAL INTRODUCTION

Humans are usually exposed to fungi and their mycotoxins produced via ingestion of contaminated food, contact and inhalation. The houseflies, *Musca domestica*, are best known as house infesting flies and also known to act as biological vector for various pathogenic agents, including the ones that affect humans (Sukontason *et al*., 2000; Skovgard and Jespersen, 2000). The housefly has the potential for dissemination of microorganisms in the environment that are associated with animal faeces and manure. These flies have been shown to feed on secretions and other human wastes, making them ideal carriers for transmitting several pathogenic microorganisms. Vectors like insects, especially houseflies, have been reported as carriers of yeast and filamentous fungi (Zarrin *et al*., 2007). Filamentous fungi are ubiquitous in nature and unavoidable (Greenberg, 1973; Barson *et al*., 1994; Hald *et al*., 2008; Banjo *et al*., 2005). However, fungi produce many secondary metabolites that include mycotoxins; thus, it is difficult to obtain definitive information as to the specific metabolites produced by each kind of fungus. In addition a variety of bacterial diseases are disseminated by housefly, which include typhoid fever, cholera, staphylococcal food poisoning (caused by *Staphylococcus aureus*) and Shigellosis and this causes problems as, unfortunately, houseflies have adapted to living in buildings associated with humans (West, 1951; Fotedar, 2001). Hence when flies are found in human structures, they are almost always considered nuisance pests and are a potential threat to human health.

Secondary metabolites are biosynthesized by fungi and some of them are toxic to humans and animals and are known as mycotoxins. Adverse health effects of fungi and mycotoxins have been recognized for centuries following environmental exposures (Hardin *et al*., 2003; Anyanwu, 2004; 2008). There are five genera of mycotoxins producing filamentous fungi that are important in food commodities: *Aspergillus*, *Penicillium* and *Fusarium* (Yiannikouris and Jouany, 2002), *Alternaria* (Dutton and Kinsey, 1996; D'Mello and Mcdonald, 1997; Smith, 1997) and *Claviceps* species (spp.), (Chu, 2002; O'Brien and Dietrich, 2005). Their occurrence and mycotoxin production varies from region to region depending on the climatic conditions (temperature and moisture) (Devegowda *et al*., 1998; CAST, 2003). The most common routes of exposure to fungi

and mycotoxins are by spore or metabolite ingestion of contaminated foods, dermal, respiratory, and parenteral routes, the last being associated with drug abuse (Peraica and Domijan, 2001). Normal building materials and furnishings are not considered to be susceptible to fungal contamination and cannot provide ample sources of nutrition for many species of moulds. It is, however, accepted that under favourable conditions, fungal spores can grow well and be amplified indoors especially when there is an adequate supply of moisture (Christensen and Sauer, 1982; Hardin *et al*., 2003). The structurally diverse mycotoxins can be carcinogenic, mutagenic, teratogenic, oestrogenic, haemorrhagic, immunotoxic, nephrotoxic, hepatotoxic, dermotoxic, and neurotoxic to humans and other mammals (Wogan, 1992; Dirheimer, 1996; de Groene *et al*., 1996; Chu, 1998; 2002; Bhatnagar *et al*., 2002). To minimize the harmful effects of mycotoxins on humans, strict regulations need to be established worldwide, especially in the rural areas of South Africa where fungal contaminated food are consumed on daily bases.

Houseflies inevitably encounter mycotoxins in nature when they feed on unharvested fruits in orchard situations and contaminated food. Houseflies can be vectors of fungal spores and also facilitate fungal access to their host plants. This interaction is manifested in many crops and increase in mycotoxin contamination. Control of houseflies can thus reduce fungal toxin levels in crops. However, understanding how houseflies promote the spread of fungi can help control of both house pests and mycotoxin contamination (Guodong, 2010). Mycotoxins attract worldwide scientific, political and economic attention because of the significant economic losses associated with negative impacts on human health, animal productivity and international trade (Paterson, 2006). The degree to which aspects such as immune suppression contribute to the overall burden of infectious disease is difficult to quantify, but is indisputably significant. Thus, food safety remains an important opportunity for addressing current health problems in developing countries (Shephard, 2008a), such as South Africa whereby information on the carriage of pathogenic microorganism including fungi by houseflies is scanty. It is in this regard that this proposed project was designed, to alleviate fungal dissemination and their derived by-products in foods and feeds intended for human consumption. It therefore becomes very crucial to conduct research and to improve knowledge on filamentous fungi and their mycotoxins produced.

1.2 PROBLEM STATEMENT

In the history of human development it is undoubtedly that house flies have been a nuisance to both man and animal throughout. However, because of their tendency to frequent pathogen-rich filth, there is a necessity to investigate the potential role of houseflies in disseminatation of fungi and in contamination of food commodities with mycotoxins.

1.3 HYPOTHESIS

Houseflies are the commonest and most familiar insects which have increasingly overcrowded human dwellings, particularly in rural areas and constitute a health hazard as well as an irritation. As they move back and forth by feeding and breeding on food commodities and filth, this may lead to pick up and the spread of diseases-causing microorganisms and the spread of fungi producing mycotoxins mechanically, thus frequent exposure to these houseflies will have an important impact on humans and their health.

1.4 AIMS AND OBJECTIVES OF THE STUDY

- To screen for fungal contamination present in houseflies, maize, porridge, bio-wipes and water samples from rural population in the Gauteng Province of South Africa.
- To evaluate the toxigenic potentials of the fungi isolated from all samples.
- To extract and identify the mycotoxins from contaminated houseflies, maize, porridge, bio-wipes and water samples.
- To determine fumonisin production by *Fusarium* spp. isolated from contaminated houseflies, maize, porridge, and water samples.
- To estimate human exposure to fumonisins from biowipe data
- To quantify the mycotoxins produced by the fungal spp. isolated.
- To determine the cytotoxic effects of mycotoxin extracts obtained from maize, porridge, houseflies and water samples.
- To recommend possible remedial action and information to the rural farmers in order to reduce houseflies population and human exposure to them.

CHAPTER TWO

2.1 LITERATURE REVIEW

Houseflies contaminate foods, especially in developing countries where there is a lack of food safety and quality is subsumed by food insufficiency. Food contamination is a major problem throughout the world and one of the route to contamination is through fungal dissemination by houseflies. Food commodities such as maize and maize-based products are often vulnerable to fungi and mycotoxins. Exposure to these contaminants presents a hazard to human health, therefore, it is imperative for continuous research on fungal dissemination by houseflies with subsequent mycotoxin production.

2.1.1 HOUSE FLY: DESCRIPTION, ORIGIN AND LIFE CYCLE

It is a well-known fact that houseflies in the order Diptera, which means "two wings", belonging to the family of *Muscidae*, are widely dispersed cosmopolitan pests in and around human homes and farms (Sanchez-Arroyo, 1998; Lecouna *et al*., 2005; Triplehorn and Johnson, 2005). As flies are now associated with human populations, they are eminently suited as a major factor for the transmission of fungi and several serious diseases (Graczyk *et al*., 2001; Zurek and Gorham, 2008). Transmission occurs when the houseflies come into contact with people or their food. Houseflies were first recorded in 1758 by Linnaeus and are thought to have evolved during the Permian period of the Palaeozoic era (Lambrecht, 1980). They now occur in abundance all the year round virtually in every region of the globe that humans exist, in all climates from tropical to temperate, and in a variety of environments ranging from rural to urban. The only exception is in areas with high altitudes and polar regions, which are prone to extreme cold temperatures (Merchant *et al*., 1987). However, the exact origin of house flies may never be known, but have been speculated by many to originate in the Middle East area of the Palaearctic region (Skidmore, 1985, Pont, 1991). It is always regarded as a sign of insanitation when flies breed in places associated with human excreta, decaying fruits and vegetables, rubbish dumps containing organic matter, ground where liquid wastes are spilled and they also have adapted well to feeding on garbage, so they are abundant almost anywhere people live (Amano, 1985; Koehler *et al*., 1998; Hogsette *et al*., 1993).

Although houseflies tend to live close to humans, they do not bite and are attracted to food by sensory receptors for smell and taste distributed on their legs and feet, and also on their mouthparts (Moon, 2002; Nazni *et al*., 2005). They cannot eat solid food, to do so they vomit on solid food which allows digestive enzymes to liquefy the food and make a solution of it to ingest in a liquid state (Zarrin *et al*., 2007; Vasan *et al*., 2008). As houseflies vomit frequently, the vomit drops from the houseflies' stomach contents is often a rich culture of disease agents and infect food by transporting microorganisms on the body, leg hairs, and the sticky parts of the feet (Olsen, 1998; Pandian and Asumtha, 2001; Thaddeus *et al*., 2001; Clavel *et al*., 2002). In addition, houseflies ingest microbes with their food and not only microbes not only reside temporarily in their digestive tract but can also proliferate, be disseminated and deposited to whatever various sites they happen to land on through feeding and defecation (Sasaki *et al*., 2000; Kobayashi *et al*., 2002). This contributes to the trail of fungal contamination left behind on the surfaces of fresh food and water which are eventually ingested by people. Houseflies, in spite of their role as pathogen carrier and overall irritation they also perform some useful functions. They act as scavengers to help consume dead animals and rotting waste, serve as food for spiders and birds, and most importantly they are effective pollinators. They are known to be "incidental" pollinators. As they move around plants in search of nectar, they transfer pollen on their body hairs from one flower to another (Wilson *et al*., 1991).

Houseflies undergo complete metamorphosis, however, the life cycle (Figure 2.1) begins with an egg, then develops through a larva phase to a pupa phase, and finally into an adult stage in their development (Krafsur *et al*., 1985; Panabang, 2012). During mating male houseflies usually chase down female counterparts to fertilize before she can lay her eggs. Unlike nesting insects, houseflies are solitary creatures, males and females do not stick together after mating. Female houseflies do not protect their eggs, instead they leave their eggs where they will be safe from predators and have plenty to eat upon hatching. The number of eggs laid individually by a female housefly at one time is undoubtedly large, totalling up to 500 eggs batches of 75 to 150 in a three to four day period (Moon, 2002; Bennett, 2003).Typically, the eggs are laid on any suitable food source such as manure, garbage, exposed faeces of any type, decomposing animal carcases and rotting food including human food, where a high degree of heat is maintained in order to hatch successfully (Lysyk, 1991; 1993; Lamb, 2008). The bacteriological fermentation that goes on

within the suitable food sources mentioned above usually preserves the temperature high and fairly independent of external changes so that development proceeds rapidly (Nayduch *et al*., 2002). The eggs hatch after 8 to 24 hours (hrs) and maximum egg production occurs at intermediate temperatures from 25 to 30°C. Frequently, several houseflies will lay their eggs in close proximity, leading to large masses of larvae. The eggs are white, individually resemble grains of rice bluntly rounded banana-shaped with two rib-like thickenings down each side, about 1.2 millimetre (mm) in long and often lain in clusters. In general the eggs widen in size posteriorly to anteriorly with two longitudinal dorsal surfaces and curved ridges that narrow prior to reaching the caudal end (De Bartolo, 1986; Sanchez-Arroyo and Capinera, 2008). House flies have three larval instars merging from the split egg case, the larva also known as maggot. The larva is semi-transparent maggot with no eyes, later becoming white with twelve visible segments (Fig. 2.1), becoming larger at the posterior end. The tiny head is usually pulled back into the anterior segments, which progressively becomes more chitinized and crescent-shaped through moults.

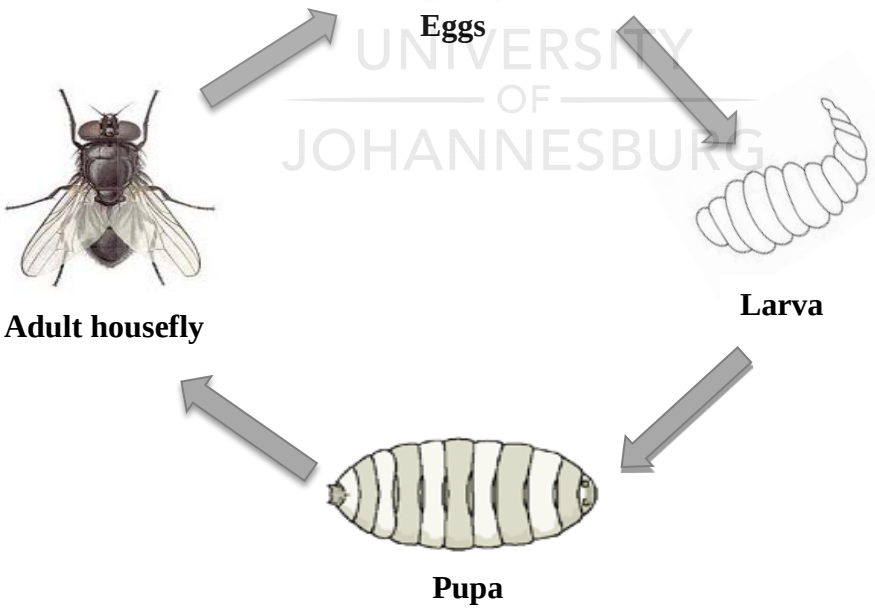


Figure 2.1 Life cycle of housefly, (Panabang, 2012).

Additionally, the first and second stage larvae have two spiracular openings used for gas exchange and a third opening appears on the third instar larvae (Hogsette, 1995; Banjo *et al*., 2004). Two black, hook-like teeth projecting from above the mouth are used for movement through the breeding place and for tearing up potential food. The larva has no legs, but on the lower sides of segments six to twelve are crescent-shaped pads bearing short spines which assist its movement. After the larval instars, legless fully grown larvae emerge, about 7 to 12 mm in length, with a greasy and creamy coloured appearance. High moisture from food substrate favours the survival of the houseflies' larvae. This stage of larval growth may take 3 to 30 days and the optimal temperature for larval development is 35 to 38°C, though larval survival is greatest at 17 to 32°C (Sharififard *et al*., 2011).The nutrient rich substrates in which the eggs were laid on provide excellent developmental substrates which the larvae immediately feed on, taking in only fluids and tiny particles to fully develop. Although larvae are developed with no compound eyes they are able to distinguish light and darkness since they move away from light. This negative response to light tends to keep them in the warmer, moister regions of its breeding ground. At the end of their third instar, its reactions to light and moisture change, the larvae crawl to a dry, cool place just below the surface of the breeding ground and emerge into the pupa stage of its life (Hogsette, 1995; Moon, 2002; Sanchez-Arroyo and Capinera, 2008; Panabang, 2012).

During pupation, the larvae cuticle are not cast but retained as a pupae case also known as puparium, about 8 mm long. At the beginning of pupation, the pupae vary from yellow to white in colour but the latter gradually darkens and hardens, becoming reddish brown as the pupae ages. The puparium are medially enlarged with bluntly rounded ends. Two pupae horns are located laterally just prior to the posterior boundary of the first abdominal segment (Siriwattanarungsee *et al*., 2005). Posterior spiracles are located on the posterior end and appear as two flat, circular prominences. The anterior spiracles are situated on the puparium in the same location as in the third instar larvae. A sac-like structure, the ptilinum, in the head, can be blown out by blood pressure and by this means the top of the puparium are burst open, after which the ptilinum are withdrawn into their head. Both females and males houseflies upon emerging from the puparium, crawl their way upward through the surface of the breeding ground where their wings expand and the exoskeleton hardens, dries and assume their normal coloration in the next

few hours, then they fly away. However, this pupal stage usually takes four to twenty days depending on temperature and humidity (Madeira, 1998; Skovgard and Steenberg, 2002; Siriwattanarungsee *et al*., 2005).

Similarly to all flies, the structure of houseflies has two wings and three distinct divisions into head, thorax and abdomen with hair-like projections covering the majority of their body. Their head have red compound eyes and sponging mouthparts. Additionally, their thorax is grey, bears four equally broad dark longitudinal stripes on the dorsum and a sharp upward bend in the fourth longitudinal clear wing vein (West, 1951). Their abdomen has yellowish with dark midline and irregular dark markings on the sides, their posterior portion is brownish black and a dark longitudinal line extends along the middle of the dorsum. The three pairs of legs like most of the rest of the body, blackish brown in colour, covered with sensory setae, and the last tarsus on each leg bears a pair of claws and two glandular pads which secrete a sticky substance supporting them to walk on smooth vertical surfaces (Greenberg, 1973; Jiang *et al*., 2002). Adult houseflies are only about 8 to 12 mm long, with the female usually slightly larger than the male (Fig. 2.2). Furthermore, it is possible to distinguish between the males and females apart by their size. The females have a greater space between their two red compound eyes and while in males, the eyes almost touch (Triplehorn and Johnson, 2005).

Figure 2.2 Female (Left) and Male (Right) Houseflies (Mullens *et al*., 2001).

Once the adult houseflies hatch from the pupae stage, they have an estimated life span of fifteen to thirty days. However, without food they may only survive about two to three days. In the study conducted by Buchan and Sohal (1981), adult males and females isolated from the opposite sex live longer than when they are grouped together. The development of an egg into an adult stage depending on the favourable conditions of temperature from 25 to 30°C, humidity

and food the entire life cycle may take from six to forty two days and the length of life is usually from two to three weeks but in cooler conditions it may be as long as three months (Buchan and Sohal, 1981). In ten to fourteen days adult female houseflies become sexually mature are able to start producing eggs after two days of life and four days after mating.

During reproduction the female houseflies usually mates once during their life, thus she is considered monogamous. The abdomen distends when the houseflies are engorged with food. Copulation can last for more than 1 h, but sufficient sperm transfer can occur in less than 10 minutes (mins) Murvosh *et al*., 1964). The sperm received from the male fly during their only copulation moves into a storage area located in the female houseflies' reproductive system called the spermatheca. This sperm storage area is accessed repeatedly by several batches of eggs that the female houseflies lay throughout their lifetime as the eggs pass down the oviduct. The male houseflies have genitalia located on their posterior end, which are mostly withdrawn when not mating. The females also have posterior sex organs; their segmented ovipositor can be retracted and extended to facilitate egg laying. Female houseflies need access to suitable protein rich food to allow them to produce eggs, and manure alone is not adequate. The female attracts a male by producing muscalure, a volatile sex pheromone (West 1951; Hogsette, 1992; Howard and Wall, 1998). The complex lobes of the male houseflies genitalia clasp onto the female's ovipositor during copulation and the sperm are injected into the female reproductive system. Prior to copulation, the male sometimes grasps the female in the air, but actual copulation takes place while resting on a surface, rather than in flight. If a copulating pair is disturbed while mating they may attempt to fly a short distance to an alternate surface. Copulation is typically completed in few minutes but may take up to 15 minutes. Oviposition commences four to twenty days after copulation and the threshold for preovipositional development is about 14°C (Zurek *et al*., 2000; Nation, 2002).

The abundance of houseflies is however determined by abiotic factors such as temperature, moisture of breeding habitat and humidity and biotic factors including parasites and predators (Connelly, 2001; Howard, 2001). Temperature is inversely proportional to the life span of adult houseflies therefore; higher temperatures reduce the life expectancy, while lower temperatures increase it. In nature, predators have a significant role in regulating fly population levels

(Seymour and Campbell, 1993; Dias, 2006). Hypothetically, housefly population would grow to astronomical numbers if there were no factors limiting their production. However, the abiotic and biotic factors inhibit this from happening.

2.1.2 HOUSEFLY CONTROL MEASURES

Houseflies are existent nuisance when flying around and therefore, it is important to get rid of them. The more commonly used control measures for houseflies are sanitation, use of traps, and insecticides. In some instances integrated fly control has been implemented, but good sanitation still remain the basic step in any housefly management (Hedges, 2004; AFPMB, 2006). Food and materials on which the houseflies can lay eggs must be removed, destroyed as a breeding medium and isolated from the egg-laying adult. Since houseflies complete their life cycle in as little as seven days, removal of wet manure is necessary to break the breeding cycle. Wet straw should not be allowed to pile up in or near buildings since, straw is one of the best houseflies breeding materials (Kaufman and Rutz, 2002). Spilled feed should not be allowed to accumulate but should be cleaned up regularly each time there is a spillage. Ordinarily, housefly control from 1 to 2 kilometres (kms) around a municipality prevents houseflies' infestations (Imai, 1985). In agricultural areas, manure can be scattered over fields so that it quickly dries and becomes unsuitable for egg and larval survival. Composting of manure can be effective if the compost is maintained properly, including regular turning. Manure may be liquefied and stored in lagoons anaerobically, though at some point the solids need to be separated (Barnard and Geden, 1993). Insecticide can also be used to treat manure, though this method is highly discouraged as it interferes with biological control of flies, often resulting in a rebound of the fly population. Commonly, insecticides, especially insect growth regulators can be fed to livestock, and residual insecticide in the manure inhibits fly breeding (Geden, 1995). In animal facilities, insecticides are often applied to the favoured resting places of adults, or bait stations established to poison adults with either solid or liquid formulations.

Additionally, with the increasing incidence of insecticide resistance of houseflies population, rising costs of insecticides and a growing public concern about the actual and potential problems associated with insecticides, interest in alternative housefly control strategies has vastly improved (Georghiou and Lagunes-Tejeda, 1991; Scott *et al*., 2000; Kaufman *et al*., 2001). The

use of fly traps may be convenient in some fly control programs if enough traps are placed correctly, and used both indoors and outdoors. Houseflies are attracted to white surfaces and to bait that release odours (Darbro and Mullens, 2004). Indoors, ultraviolet light traps collect the flies inside an inverted cone and kill them with an electrocuting grid. One trap should be placed for every 30 feet of wall inside buildings but not placed over or within five feet of food preparation areas. Recommended outdoors placement areas include near building entrances, in alleyways, beneath trees, and around animal sleeping areas and manure piles (Geden, 2005). Openings to buildings should be tightly screened with standard window screen, in a manner that will deny entrance to flies.

Since the sixteenth century, and probably long before that, houseflies have been thought to transmit diseases and toxigenic fungi. Furthermore, research and experimental work proof of this was put forward, and the lists of fungi and diseases which can be carried by houseflies have been growing ever since. For this reasons and the fact that the houseflies is a capable and well-known insects, it is imperative to make a close study of its life history, habits, and also the toxigenic fungi disseminated.

2.1.3 FUNGI ASSOCIATED WITH HOUSEFLIES

Since the sixteenth century, and probably long before that, houseflies have been recognised as carriers for a wide variety of fungal species (Forester *et al*., 2009). Their primary mode for the transference of fungal spores is by collecting spores on their legs, mouthparts and the small hairs that cover their bodies. If later the flies alight on food for human consumption, harmful fungal spores are deposited on it (De Jesus *et al*., 2004). Some of the fungal spores adapt to the desiccated and relatively nutrient-poor conditions and survive in similar niches on growing, stored crops and exposed foodstuffs (Reddy *et al*., 2009). Generally, the amount of available water in the feed matrix determines whether the spores will grow or survive. For example, some fungi, are adapted to the low amount of available moisture and grow actively within stored seeds and grains. Others will produce spores or enter survival state until the moisture conducive for mycotoxin production (Frisvad and Samson, 1991; Pitt and Hocking, 1997c; Flannigan and Miller, 2001; Hussein and Brassel, 2001; Pitt and Hocking, 2009). Experiments in which houseflies have been allowed to walk over culture media in sterile dishes have resulted in the

growth of over 100 fungal colonies from fungal spores which the flies deposited (Banjo *et al*., 2005). Many of these spores are harmless to humans but others may cause serious diseases. Research on the association of fungi and houseflies is scanty however, in a study conducted by Sales *et al*. (2002) revealed a prevalence of the genus *Aspergillus*, followed by the genus *Penicillium*, *Fusarium*, *Cladosporium* and *Alternaria*. Srivoramas *et al*. (2012), approximately 57 fungal isolates were identified from the external surfaces of the houseflies comprising twelve different fungal groups. The most prevalent of the fungal groups being, *Mucorales*, *Yeast*, *Aspergillus*, *Penicillium*, *Gliocadium*, *Fusarium*, *Drechslera*, *Scedosporium*, *Cladosporium* and *Nocardia*. Apprehensions about food-borne human illnesses have led to a number of published works on the association of fungi and houseflies by Steinhaus (1946), Kaaya and Okech (1990), Norberg *et al*. (1999), Banjo *et al*. (2005), Zarrin *et al*. (2007). Hence, this study was conducted to fill the gap between the need of awareness of houseflies and food safety. The proceeding sections will deliberate on some of the literature with respect to contamination of food and feeds by fungi and mycotoxins.

2.2 FUNGI

2.2.1 OVERVIEW

Fungi are a member of a large group of eukaryotic organisms with a cell wall like plants, but they do not have chlorophyll and are therefore incapable of photosynthesis. They are major plant and insect pathogens found in just about any habitat but frequently live on the land, mainly in soil and on plant material (Prescott *et al*., 1996). Fungi are not able to ingest their food like animals do. Instead, fungi feed by absorption of nutrients from the surrounding environment. They accomplish this by growing through and within the substrate on which they are feeding (Ingold and Hudson, 1993). Fungi are divided into two big groups: yeasts and moulds. Yeasts are solitary rounded cell forms that reproduce by making more rounded forms through mechanisms such as maturing. Moulds, on the other hand, have bodies composed of thread-like long cells called hyphae. Hence, moulds are also known as filamentous fungi. The filamentous cells are connected end-to-end and grow in a branching fashion forming a network called mycelium. The mycelium that grows over and within a substrate that is used as a source of nourishment is called vegetative mycelium. In the life cycle, the vegetative mycelium may give rise to a large

organized reproductive structure called fruit body, which bears the spores and is produced solely for the release of spores (Penalva *et al*., 2002; Redecker *et al*., 2006). The discipline of biology devoted to the study of fungi is known as mycology. Mycology is the branch of biology concerned with the systematic study of fungi, including their genetic and biochemical properties, their taxonomy, and their use to humans as a source of medicine, food, and psychotropic substances consumed for religious purposes, as well as their dangers, such as poisoning (Struck, 2006).

Abundant worldwide, most fungi are inconspicuous because of the small size of their structures, and their cryptic lifestyle in soil, on dead matter and as symbiosis of plants, animals, or other fungi. They may become noticeable when fruiting as moulds and therefore, perform an essential role in the decomposition of organic matter and have fundamental roles in nutrient cycling. They have long been used as a direct source of food, such as mushrooms, in fermentation of various food products, such as wine, beer, and soy sauce. Since the 1940s, fungi have been used for the production of antibiotics, and, more recently, various enzymes produced by fungi are used industrially and in detergents (Schlegel, 1993; Joseph *et al*., 2008). Fungi are also used as biological pesticides to control weeds, plant diseases and insect pests. Many species produce bioactive compounds called mycotoxins, such as alkaloids and polyketides, which are toxic to animals including humans (Schardl *et al*., 2006).

Mycotoxins are mainly formed by certain filamentous fungi belonging to the genera *Aspergillus*, *Penicillium*, *Fusarium*, *Alternaria*, *Claviceps and Stachybotrys* spp. Of these genera, the first three are the major contributors of fungal spoilage and mycotoxin production in food supply (Barkai-Golan, 2008a*;* Pitt and Hocking, 1997a). Although a large number of different mycotoxins exist, only a few of them, namely fumonisins (FBs), patulin, aflatoxins (AFs), ochratoxin A (OTA) and *Alternaria* toxins are frequently found in food commodities (Drusch and Ragab, 2003). Among mycotoxins, AFs, OTA and FBs are of major concern, given their high occurrence and toxicity. Fungal diseases can lead to significant monetary loss due to their impact on human food supplies, depletion of nutritive value and health related problems (Blackwell *et al*., 2009). To understand the health effects of mycotoxins, however, one needs to understand the basic ecology of fungi and how mycotoxins are produced.

2.2.2 NATURAL OCCURRING TOXIGENIC FUNGI

Toxigenic fungi are microorganisms capable of producing mycotoxins (Kubatova *et al*., 1999). Human exposure to toxigenic fungi may result from consumption of plant-derived foods that are contaminated with toxins, the carry-over of toxigenic fungi and their metabolites in food commodities such as maize (CAST, 2003) or exposure to air and dust containing these fungal toxins (Jarvis, 2002). Toxigenic moulds are known to produce a large number of toxic secondary metabolites. It is well established that not all moulds are toxigenic and not all secondary metabolites from moulds are toxic. Mycotoxins are formed mostly by certain filamentous fungi, and the most important are among others, species *Aspergillus*, *Alternaria*, *Claviceps*, *Fusarium*, *Penicillium* and *Stachybotrys*, which may grow on a number of food commodities (Reddy *et al*., 2009). The purpose of this review is to summarize the current knowledge about these important genera of toxigenic fungi, which may have potentially adverse effects on human health.

2.2.2.1 *Aspergillus* species

The genus *Aspergillus* is a large genus of filamentous fungi characterized by a unique spore bearing structure. They are classified under the genus of hyphomycetes characterised by their distinctive conidiophore with large, heavy walled stipes with a swollen apices termed vesicles (Fig. 2.3). They reproduce by forming mitotic spores (conidiospores) at the end of the conidiophore. Production of these spores is often phenomenal, making *Aspergillus* one of the most common fungi on earth (Pitt and Hocking, 1997b; Klich, 2002a; 2009). Identification of *Aspergillus* species has conventionally dependent on macroscopic colony characteristics and microscopic morphology. Over 180 species have been described and a number of these result from changes in species concepts based on molecular and physiological data, and several of these new species cannot be distinguished morphologically (Klich, 2009). *Aspergillus* spp. are amongst the most common moulds that are frequently encountered by humans and are the aetiologic agents for a remarkably diverse set of human diseases (Park and Mehrad, 2009).They are most abundant and widely distributed in soil, water, air, seed and food (Anaissie and McGinnis, 2003) and some of them are associated with allergic broncho-pulmonary disease, mycotic keratitis, otomycosis, nasal sinusitis and invasive infection (Diba *et al*., 2008).

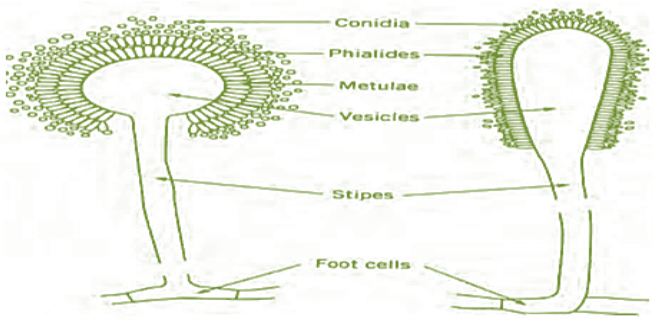


Figure 2.3 Distinctive structures of *Aspergillus* species, (Klich, 2009).

Although more than 180 species have been recognized *Aspergillus* spp., *A. flavus*, *A. fumigatus* and *A. terreus* still account for the vast majority of human pathogen. Other species including *A. niger*, *A. nidulans*, and *A. ustus* are rarely encountered as causes of invasive disease (Sampson, 1994; Blackwell *et al*., 2005). *Aspergillus* spp. are used in the fermentation industry, but they are also responsible of various plant and food secondary rot, with the consequence of possible accumulation of mycotoxins. The aflatoxin producing *A. flavus*, *A. parasiticus*, *A. niger*, *A. ochraceus* and *A. carbonarius* spp. are frequently encountered in agricultural products (Hedayati *et al*., 2007; Perrone *et al*., 2007).Various mycotoxins still continue to be recognized in foods and feeds contaminated by *Aspergillus* spp., the most significant are the aflatoxins and ochratoxin A (Varga *et al*., 2004). In addition, *Aspergillus* spp. such as *A. sclerotiorum*, *A. ostianus*, *A. alliaceus*, *A. carneus*, *A. oryzae*, *A. melleus*, *A. tamarii*, and *A. versicolor* remain recognised to cause diseases in economically important crops and to produce potent mycotoxins (Klich, 2002b).

2.2.2.2 *Fusarium* species

Fusarium spp. are also a diverse genera categorized under phylum Ascomycota and are ubiquitous fungi that are distributed worldwide in soil, from the temperate to the tropical regions (Leslie and Summerell, 2006). *Fusarium* spp. are commonly reported as endophytes, saprophytes, and pathogens particularly in economically important crops (Nelson *et al*., 1983; Burgess *et al*., 1994). This ubiquitous fungus exists in soils and in association with plants and plants products, its conidia (Fig. 2.4) are water-borne, but in some instances may be airborne and its chlamydospores are typically soil-borne (Smith, 2007). Some pathogenic spp. also produce

mycotoxins that contaminate cereal crops that are associated with human and animal diseases if they enter the food chain.

Figure 2.4 Sketch of conidia produced by *Fusarium* species (Smith, 2007).

Fusarium spp. may cause a wide range of plant infections, such as vascular wilts, root, stalk and cob rots, collar rot of seedlings, rots of roots, and corms. In the previous years, research has been directed towards an increased risk of *Fusarium* infection and the production of the associated mycotoxins in crops. (Champell *et al*., 2004; Blackwell *et al*., 2005; Vogelgsang *et al*., 2006; Maiorano *et al*., 2008). The genus *Fusarium* cause a worldwide devastating disease of cereal grains, *Fusarium* head blight (FHB), and this has a significant impact in losses of both yield and quality of cereals before and immediately after harvest (Logrieco and Bottalico, 2001; Bottalico and Perrone, 2002; Thrane *et al*., 2004). *Fusarium* head blight in cereals can be isolated and predominately produced by a number of several spp. of economic and health importance which includes *Fusarium avenaceum, F. culmorum, F. equiseti, F. graminearum, F. anthophilum, F. moniliforme, F. poae, F. tricinctum, F. sporotrichioides, F. proliferatum, F. oxysporum* and *F. verticillioides* (Bottalico and Perrone, 2002; Kosiak *et al*., 2003; Mackinaite *et al*., 2006). In addition to being plant pathogens *Fusarium* spp. are well known to produce potent toxins, which include trichothecenes (deoxynivalenol (DON), nivalenol (NIV), T-2 toxin, and HT-2 toxin) (Park *et al*., 1996; Doohan *et al*., 2003; Llorens *et al*., 2006), Zearalenone (ZEA) in the Transkei region of South Africa (Marasas and Nelson, 1987) and FBs (Gelderblom *et al*., 1988; Moss, 1992; Voss *et al*., 2001; Dutton, 2009).

2.2.2.3 *Penicillium* species

Penicillium is a large well-known genus of moulds, frequently found on produce such as cereals and comprises over 200 spp. with a worldwide distribution to be linked with storage diseases of plants (Bragulat *et al*., 2008; Hyang, 2012). *Penicillium* spp. produces paintbrush-like heads and stalk called conidiophore, while the end of each branch is a well-defined cluster with spore-producing cells, phialides. Chains of spores are usually formed from the tip of each phialide; these spores in *Penicillium* frequently contain blue and green pigments which normally give the colonies on foods and feed their characteristic colour (Fig. 2.5) (Larone, 1995; St-Germain and Summerbell, 1996; Bancerz *et al*., 2005). Different spp. of these fungi produce several types of secondary metabolites, ranging from the antibacterial drug penicillin to the antifungal drug griseofulvin, along with many compounds that are toxic to humans and animals. The most common identified in a variety of food commodities include *P. chrysogenum*, *P. citrinum*, *P. janthinellum*, *P. marneffei*, *P. purpurogenum*, *P. expansum*, *P. echinulatum*, *P. oxalicum*, and *P. solitum* and are capable of causing spoilage on fruits and cereal products (Pitt and Hockings, 1997a; de Hoog *et al*., 2000; Frisvad and Thrane, 2002; Kim *et al*., 2002). Some plant derived *Penicillium* spp. not only damage plants but also produce harmful toxins, such as citrinin (CIT), cyclopiazonic acid (CPA), patulin (PAT), penicillic acid (PIA) and roquefortine C (RQC) (Larsen *et al*., 2001; Bernhoft *et al*., 2004; Bouhet and Oswald, 2005) and these will be discussed into detail further in this section.

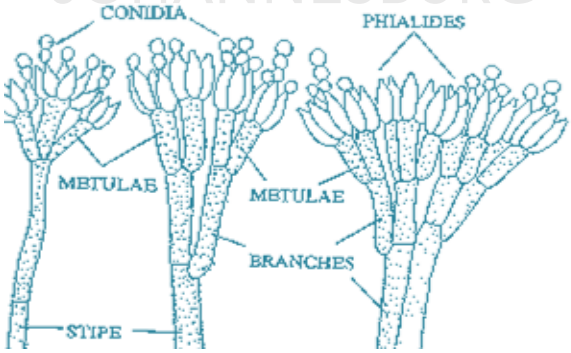


Figure 2.5 Different types of branching conidiophore in *Penicillium* species, (Samson *et al*., 1984).

2.2.3 OTHER NATURAL OCCURRING FUNGI

2.2.3.1 *Alternaria* species

Alternaria is cosmopolitan and ubiquitous in nature, known as major plant pathogens commonly growing on organic materials, particularly in grains and grain-based products (Pritchard and Muir, 1987; Brandt and Warnock, 2003). They are prevalent in both humid and semi-dry regions and can contaminate growing plants in the field. The spores of *Alternaria* spp. are often beaked, always multi-celled, pigmented and they are produced in dark branching chains (Fig. 2.6). The cells are divided longitudinally and transversely which give a distinctive appearance that makes them easy to recognize (Larone, 1995; St-Germain and Summerbell, 1996; Collier *et al*., 1998).

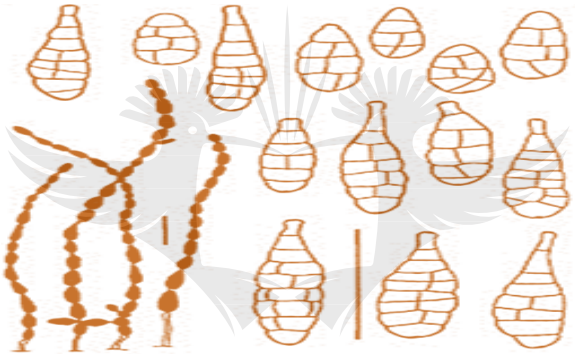


Figure 2.6 Morphological structure showing spores of *Alternaria,* (Taralova *et al*., 2011).

Alternaria is an opportunistic pathogen and can cause disease in both humans and animals (Mayser *et al*., 2002; Barkai-Golan, 2008b). As *Alternaria* metabolizes, it produces tenuazonic acid, alternariols, tentoxin, altertoxin I and other mycotoxins which can cause allergic reactions and potentially cause diseases (Andersen *et al*., 2002). At least 20% of agricultural spoilage is caused by *Alternaria* spp. and most severe losses have reached up to 80% of yield (Nowicki *et al*., 2012). Hence, it has been suggested that in some areas in China *Alternaria* toxins in grains might be responsible for oesophageal cancer (EFSA, 2011). Due to their potential harmful effects and their mycotoxin profile, *Alternaria* toxins are of concern to public health.

2.2.3.2 *Stachybotrys* species

The *Stachybotrys* are filamentous fungi belonging to the family of *Dematiaceae* and frequently isolated as contaminants from water damaged materials, nature and indoor environments (Fog, 2003; Castlebury *et al*., 2004). They are cellulolytic saprophytic fungi also with worldwide distribution, found in soils and substrates rich in cellulose including straw and cereal grains. This genus contains about 15 spp., some of which are important pathogens (Pinruan *et al*., 2004; Kirk *et al*., 2008). *Stachybotrys* produces cottony, rapidly growing colonies with septate hyphae, conidiophores, phialides and conidia observed when viewed under the microscope (Fig. 2.7). The hyphae and the conidiophores of *Stachybotrys* fungi appear to be hyaline initially and become darkly pigmented with age. The conidiophores are branched and bear phialides at their tips. These phialides are cylindrical in shape with swollen upper portions. The conidia are oval, pigmented, 1-celled, and in 3 to 10 clusters (Larone, 1995; Haugland *et al*., 2001).

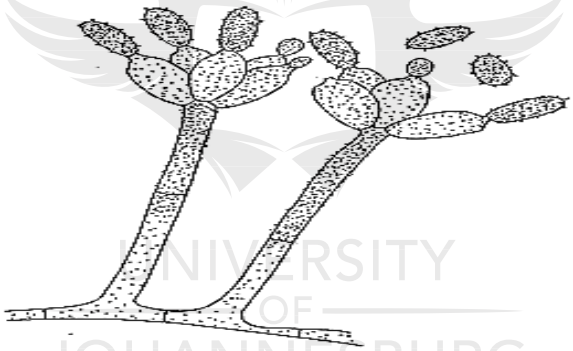


Figure 2.7 Microscopic features of *Stachybotrys* fungi, (Larone, 1995).

Similar to various genera of filamentous fungi, *Stachybotrys* has been known to produce a variety of extremely toxic metabolites such as verrucarins, roridins, satratoxins, cyclosporines and trichothecenes. However, not all *Stachybotrys* species produce trichothecenes and some of these fungi lose the ability to produce under certain conditions (Burge and Ammann, 1999; Nakumura *et al*., 1995; Pitt, 2000a; Tuomi *et al*., 2000). *Stachybotrys* is also another fungus that has the ability to produce mycotoxins suspected to be carcinogenic and cause immunosuppression (Corrier, 1991). It has attracted public attention to its effect on human health following reports of its association with idiopathic pulmonary haemorrhage in infants (Dearborn *et al*., 1999; Vesper *et al*., 2000; Tripi *et al*., 2000). These toxins may be acquired by ingestion of

food products contaminated with the fungus, via direct inhalation of the spores and may be absorbed through an individual's skin and eyes. Eventually the mycotoxins find their way into the blood. In addition to these toxins exposure may have a wide range of effects depending on the volume and the length of exposure (Pitt *et al*., 2000a; Sudakin, 2000; CDC, 2001). The actual role of *Stachybotrys*e in development of human disease is however, poorly defined and is still not known whether it is similar to the other mycotoxin-producing fungi with respect to its pathogenic potential. Nevertheless, the public must continue to be concerned about health risks resulting from exposure to mycotoxins produced by *Stachybotrys* and other fungi present in indoor air.

Furthermore, human diseases associated with detrimental toxins produced by these fungi have been described and studied in depth for centuries. Typical examples are poisoning from food contaminated with fungi such as the *Aspergillus*, *Fusarium*, *Penicillium*, *Alternaria*, *Acremonium*, *Stachybotrys*, *Claviceps*, *Cladosporium*, *Bipolaris* and *Aureobasidium* as some of them are described above. Fungal infestation and subsequent mycotoxin production may occur during plant growth, maturity, harvesting, storage, and processing of grains. As in any case, these genera of fungi require certain environmental conditions which play a substantial role in their survival and production of toxic metabolites. Therefore, the relative significance of each of the factors in fungal growth will be summarized in this section.

2.2.4 FACTORS INFLUENCING FUNGAL AND MYCOTOXIN PRODUCTION

Fungal colonization and growth are generally influenced by variety of conditions. These conditions include nutrients, temperature, pH, water activity (a_w), light and oxygen (O_2) (Domsch *et al*., 1993; Northolt *et al*., 1995). According to Frisvad and Samson (1991), conditions for mycotoxin production are generally more restrictive than those for growth and can differ between different mycotoxins produced by the same species and between fungi producing the same mycotoxin.

Of these above mentioned factors, a_w is perhaps the most important factor affecting colonisation of fungi. All fungi require moisture for growth but the amount required varies widely. Availability of moisture allows for the diffusion of extracellular digestion of nutrients outside the fungal cell and for the enzyme activity internally. Although each fungus has its own optimal and

minimum moisture requirement, moisture determines whether fungi can colonize a substrate or not (Moore-Landercker, 1996; Flannigan and Miller, 2001). Fungi that are capable of growing at very low water activity are referred to as xerophiles, for example *Aspergillus* (*A. penicillioides*) and *Penicillium* (*P. chrysogenum*) spp. (Pitt and Hocking, 2009). Those that are capable of growing at very high water activity are referred to as hydrophilic, *Stachybotrys*, *Chaetomium* and *Alternaria*. *Penicillium* and *Aspergillus* are identified to grow best at an a_w of 0.95, whereas *Fusarium* grow best at a higher a_w of 0.98 (Moss, 1996).

Fungi are heterotrophic, they use preformed complex organic molecules available in their environment for their sources of hydrogen, phosphorus, carbon, nitrogen, sulphur and potassium for biosynthesis and energy sources. Some of the essential requirements include vitamins, especially biotin and thiamine and micro-elements such as copper, iron and zinc (Russell *et al*., 1991; Moore-Landercker, 1996). Nutrient requirements for moulds may vary from fungi to fungi. Some fungi may thrive well on substrates with high sugar and salt content. Some may prefer simple sugars while others have the ability to utilize complex sugars (Hussein and Brassel, 2001).

Temperature is also a factor required, even though majority of fungi are mesophilic. They can grow at temperatures within the range of 10-35^{o}C and optimum temperatures for growth may range between 15 and 30^{o}C (Pitt and Hocking, 1997c). It has been found that *Penicillium* spp. have a lower temperature range for optimal growth and mycotoxin production than *Aspergillus* spp., which may be 25-30^{o}C and 30-40^{o}C for *Penicillium* and *Aspergillus* (Robert and Raymond, 1994). Various *Fusarium* spp. have lower optimal temperature of 8-20^{o}C, for growth and reproduction. Several fungi are psychrophilic and are unable to grow above 20^{o}C. Thus, a significant number are psychro-tolerant and are able to grow at room temperature and at freezing point (Robert and Raymond, 1994).

Light is an important signal for every living cell. In contrast to plants, fungi use light as a source of information but not as a source of energy (Tisch and Schmoll, 2010). Furthermore, since optimal adaptation to both the harmful and beneficial effects of light significantly enhances fitness of an organism, it can be considered crucial for successful competition and survival in

nature. Many fungi are capable of sensing light to recognize and anticipate conditions unfavourable for vegetative growth, such as deprivation of nutrient on the soil surface and loss of water (Corrochano and Avalos, 2010). On the other hand, the same signal is needed for appropriate timing of production and dissemination of conidia, which happens on the surface, in light. Though, many fungal species grow well in the dark, some prefer daylight and often need alternate light and darkness in order to produce spores (Idnurm and Heitman, 2005; Corrochano, 2007).

The influence of pH as a requirement in fungi differs, most will grow well over the pH range 3-7. Some species such as *A. niger* and *P. funiculosum* can grow at pH 2 and below (Wheeler *et al*., 1991) and at pH of 7.5 for *F. verticillioides* (Narasimha Rao *et al*., 2010). At higher pH values, fungi compete with bacteria as food spoilers. Some fungi are less affected by pH over a broad range, commonly 3 to 8 (Wheeler *et al*., 1991). However, the pH of a medium may exercise important control over a given morphogenic event without remarkably influencing the overall growth of a fungus (Pitt and Hocking, 1997c).

Fungi like all organisms require O_2 to grow and it influences the growth and production of mycotoxins. The production of patulin and penicillic acid decrease sharply at low oxygen concentrations, while fungal growth is not noticeably influenced (Northolt, 1979). Additionally, spore germination and mycelium growth of fungi have different sensitivities to both reduced and increased oxygen concentrations levels. The growth of *Aspergillus* is restricted at very low oxygen concentrations of <1% (Pitt and Hocking, 1997c).

Worldwide occurrence of secondary metabolites in plant and food materials intended for human feeding have been studied and recorded, with special attention to those that compromise human health. Nevertheless, these metabolites represent a comprehensive range of chemical structures with physiological properties. To establish the degree of exposure combined with their distribution in the human body, an overview on these toxic metabolites will be discussed.

2.3 MYCOTOXINS IN A GLOBAL AND SOUTH AFRICAN CONTEXT

Intense interest has now been enthused, however, by the mounting evidence of fungal toxicity, and research is being conducted in many parts of the world, notably China, Kenya, Japan, USA, and South Africa (Rheeder *et al*., 1992; Muthomi *et al*., 2009; Chilaka *et al*., 2012; Li *et al*., 2014). Agriculture supplies food, raw materials and generates household income for the majority of South Africans, especially in the rural areas (Dutton, 2009). Agricultural holdings are generally small and scattered; farming is often of the subsistence variety, characterized by simple tools and shifting cultivation as well as improper methods of storage and transportation of agricultural products (Akande, 2010).Therefore, it is impossible to eliminate mycotoxins from foods and feeds in spite of the regulatory efforts at national and international levels to remove contaminated commodities. Agricultural commodities have contributed significantly to the discussion about potential hazards involved and have increased in particular the awareness of mycotoxins. Safety awareness in foods and feeds production has increased due to the simple fact that methods for testing residues and undesirable substances have become noticeably more sophisticated and more available at all points of the supply chain (Zaki *et al*., 2011).

Mycotoxins comprise of a family of fungal toxins contaminating agricultural products, many of which have been implicated as chemical precursors of toxicity in humans and animals. However, agricultural products remain prone to contamination by several toxigenic fungi during the production, storage and processing stages (Pierre, 2007; Rheeder *et al*., 2009; Steyn *et al*., 2009). Fungi and mycotoxin contamination of food commodities is specific; such being that *Fusarium* species which have been reported to be more associated with maize, maize-based products (Sanchez-Hervas, 2008). The supply of high quality, healthy maize is a national priority since these commodities represent the staple food of many South Africans. In addition, maize is an important input in most food production in South Africa, a shortage in maize leads to an increase in food prices and inflation (Ncube and Flett, 2012). Formation of mycotoxins varied between species as well as within a given species. A variety of physical, chemical, and biological methods to counteract the mycotoxin problem have been reported (Moss, 1991), but large-scale, practical, and cost-effective methods for detoxifying mycotoxin-containing feedstuffs are currently available (Kensler *et al*., 2004; Burgos-Hernandez *et al*., 2002; Aroyeun and Adegoke, 2007; Jouany, 2007). However, detoxification strategies for the contaminated foods and feeds should

be done to reduce or eliminate the adverse actions of mycotoxin to improve food safety and prevent economic losses (Robens and Cardwell, 2003; Leibetseder, 2005). Aflatoxins, fumonisins, ochratoxins, zearalenone and trichothecenes (deoxynivalenol and nivalenol) are mycotoxins that are of greatest public health and agro-economic significance (Hussein and Brasel, 2001). Thus, they are a major concern in subsistence agricultural systems and in populations where maize is a staple food. Fumonisins are produced by *Fusarium verticillioides* in maize worldwide and they are present at low levels in most field-grown maize (Shephard, 2008a), but may spike to high levels depending on both the environment and genetics of the host plant. Fortunately, the aflatoxins, the most notorious group of highly potent hepato-carcinogens occur extremely rarely on South African maize (FAO, 2004). The proceeding review aims to discuss in details mycotoxin contamination of food and agricultural products, health implications of mycotoxin contamination of foods to man and animals and ways of controlling together with prevention strategy for fungal and mycotoxin contamination.

2.4 MYCOTOXINS

2.4.1 Overview: definitions and concepts

Mycotoxins are defined as secondary metabolites produced by specific fungi that contaminate food and agricultural commodities, which consist of highly toxic compounds of low molecular weight (Ratcliff, 2002; Ghali *et al*., 2008). Mycotoxins are capable of contaminating a variety of feed and food; essentially maize and maize-based products, and can appear in the food chain consumed by humans via natural route (Samson *et al*., 2002). Thus, most foods are susceptible to invasion by fungi during some stage of handling in production, processing and storage. In general, mycotoxins are produced in foodstuffs and agricultural commodities by three genera of fungi: *Aspergillus*, *Penicillium* and *Fusarium* (Piva and Fabio, 1999; CAST, 2003). However, when ingested by man, mycotoxins can cause disease and death depending on the quantity of and duration of exposure and the volume ingested (Bennett and Klich, 2003). Various cases of poisoning by mycotoxins have been known for a long time. The detection of fungi in agricultural products does not necessarily mean there is presence of mycotoxins. Its production depends on the species, substrate and environmental conditions. It is well established that not all species are toxigenic and not all secondary metabolites from fungi are toxic. Furthermore, due to their

chemical structures and classifications, it crucial to determine the occurrence of mycotoxins in food before declared unfit for animal or human feeding.

Exposure to mycotoxin, in most cases is likely to occur in parts of the world where poor methods of food processing are common, where malnutrition is below poverty level, and where regulations exist only to protect exposed populations. Nevertheless, in developing countries, specific subgroups of humans may be susceptible to mycotoxin exposure. In the Limpopo Province, for example, the populations consume more maize and maize-based commodities and their storage facilities and houses are more likely to harbour fungi at high levels (Phoku *et al*., 2012) which may involve houseflies, since they occur commonly in contact with human populations and their food (Zurek and Gorham, 2008). Currently, a great deal of interest has been generated regarding the study of toxigenic fungi and mycotoxins. To date, over 100 countries in both developed and developing countries have regulations regarding the most commonly found mycotoxins in foodstuff (Devegowda *et al*., 1998; Peraica *et al*., 1999a; van Egmond *et al*., 1989). The most common mycotoxins includes AFs, CTN, DON, FBs, Ochratoxins (OTs), PAT, Trichothecenes (TH) and ZEA, along with the fungi that produce them (Table 2.1). These are just some examples of mycotoxins of greatest public concern and agro-economic importance and research on mycotoxins in general has increased drastically (Vasanthi and Bhat, 1998; WHO, 2006; Wu, 2006).

Table 2.1 Most common mycotoxins of significant concern in agricultural commodities and the fungi that produce them, (Modified from D'Mello and Macdonald, 1997)

Fungi	Mycotoxin
Aspergillus flavus, A. parasiticus, A. nomius, A. tamarii	Aflatoxins
Penicillium citrinum, P. expansum	Citrinin
A. ochraceus, P. cyclopium, P. verrucosum	Ochratoxin A
P. expansum	Patulin
Fusarium culmorum, F. graminearum, F. sporotrichioides, F. poae	Deoxynivalenol
F. sporotrichioides, F. poae	T-2 toxin
F. sporotrichioides, F. graminearum, F. poae	Diacetoxyscirpenol
F. culmorum, F. graminearum, F. sporotrichioides	Zearalenone
F. verticillioides, F. proliferatum, F. subglutinans	Fumonisins

According to cell biologist, in addition to general toxic effects, mycotoxins may be classified as carcinogens, mutagens and teratogens based on the detrimental effect they pose on humans (Bhatnagar *et al*., 2002; Bennett and Klich, 2003). Thus, these effects are referred to as mycotoxicoses, which maybe mediated in a number of organs, particularly the kidney, liver, lungs, and the nervous, endocrine and immune systems (CAST, 2003). Furthermore, symptoms of mycotoxicoses depend on the type of mycotoxin; the concentration and the length of exposure, including the health, age, gender of the exposed individual. Figure 2.8, is a representation of some general relationships in a mycotoxicosis. Frequently, the synergistic impacts associated with genetics, foodstuff and interactions with other toxins have been poorly understood. For that reason, it is possible that lack of vitamin supply, caloric deprivation, alcohol abuse, and infectious disease status may all have compounded effects with mycotoxins (Bennett and Klich, 2003).

Additionally, mycotoxins have been linked to a variety of acute and chronic health effects (Boonen *et al*., 2012). For example humans often consume food contaminated with low levels of fumonisin, which over a period produce a chronic mycotoxicoses (Dutton, 2009). In contrast incidences of acute liver disease have been found in India following high levels of mycotoxin ingestion (Bhat, 1991). Furthermore, research studies on acute aflatoxicosis were also reported in Malaysia (Lye *et al*., 1995) and Kenya (Shephard, 2004; Lewis *et al*., 2005) following aflatoxin consumption. The outbreak of chronic abdominal pain and diarrhoea was associated with high intake of fumonisin in India (Bhat *et al*., 1997). Studies of fumonisin B_1 (FB_1) was also carried out in South Africa, with high incidences rates of oesophageal cancer (Sydenham *et al*., 1990), following consumption of maize and maize-based products. Maize is the staple food grown and consumed by rural farming communities of Africa, these populations may be chronically exposed to highly fumonisin contaminated food (Marasas *et al*., 1981). According to Chelule, (2004) fumonisin appears to be produced in association with nitrosamines, which are powerful carcinogens and hence this may explain their correlation to the prevalence of oesophageal cancer which has a high mortality rate amongst Black Africans.

In many regions of South Africa, dietary staples of rural populations, especially home-grown maize is commonly infected with high levels of fungi and mycotoxins. Regular intake of high

levels of mycotoxins is likely to have impact on human health. However, this may impute to positive correlations and a number of possible consequences including chronic malnutrition, immunosuppression to individuals infected with the Human Immunodeficiency Virus (HIV) (Turner *et al*., 2003) and impaired growth development in children (Gong *et al*., 2004).

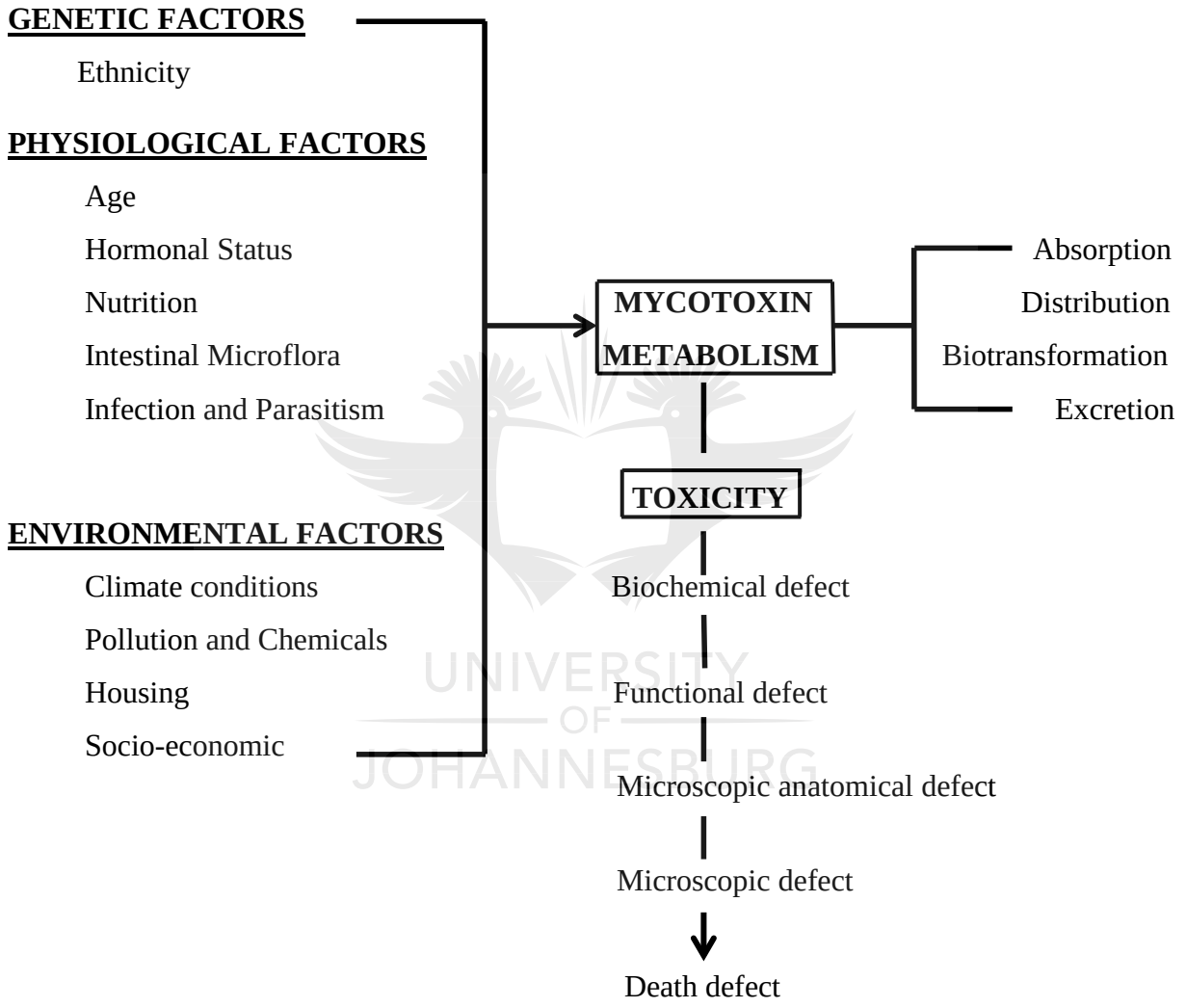


Figure 2.8 A simplified representation of some general relationships in a mycotoxicosis, modified from (Bryden, 1982).

2.4.2 Mycotoxins exposure

The human body is designed for effectively extracting nutrients from food ingested. The concern over exposure to mycotoxins and human disease dates to antiquity. Incidence of mycotoxins in

various foods across the globe is well-known, which is much more problematic in developing countries such as South Africa (Devegowda *et al*., 1998). Excessive exposure to mycotoxin-contaminated commodities can cause hazardous health effects in susceptible individuals irrespective of the type of mycotoxin and the degree of exposure (IARC, 1993a; Weinhold, 2007). With respect to exposure, only two concepts are needed to understand the negative effects of mycotoxins on human health, acute toxicity and chronic toxicity. Acute toxicity is referred to the rapid onset of an adverse effect from a single exposure. While, Chronic toxicity is the delayed onset of an adverse effect from multiple, long term exposures (Surai *et al*., 2008).The most common route of human exposure to mycotoxin is through oral, dermal and parental and inhalational route (Peraica and Domijan, 2001; Upadhaya et al., 2010). Human exposure can be via one of two routes; direct exposure due to the consumption of mycotoxins, usually contaminating plant materials, or indirect exposure through the consumption of contaminated animal products containing residual amounts of the mycotoxin ingested by the food producing animals (Pestka, 1995; Boutrif and Bessy, 2001). With regard to inhalation, when a population breathe in spores, some fungi may begin to grow in the living tissue (Muller and Seidler, 2010), attaching to cells along the lungs and create inhalation problems (Erol, 2010; Simicic and Matos, 2010). Agricultural workers, especially in the rural farming communities could be exposed at work to dust borne fungi and their products may possibly be released in large amounts into air of breathing zone during farming activities such as harvesting (Gora *et al*., 2009). The exposure could be due to the dusty nature of the substrates, high levels of spores and mycelia production ability of the fungi, especially in agricultural commodities, (Tejada-Simon *et al*., 1995). Krysinska-Traczyk *et al*. (2001) determined the levels of fungi and mycotoxins which posed potential risk of mycotoxicoses to agricultural workers exposed to grain dust when handling wheat during threshing, unloading, shuffling, and other farm occupations in Eastern Poland. They found that the majority of wheat grain and grain threshing dust contained notable quantities of fusariotoxins (moniliformin, deoxynivalenol, nivalenol). On the other hand, mycotoxins such as trichothecenes are highly toxic when they are breathed in, than when they are ingested. In most cases, since humans are exposed through consumption of mycotoxin-contaminated food. However, some foodstuffs are processed prior to consumption; to some degree most mycotoxins remain toxic even after processing (Reddy *et al*., 2010; He and Zhou, 2010). Additionally, fungal spores often produce more than one type of mycotoxins; an individual will typically be exposed

to a mixture of mycotoxin types (Fink-Gremmels, 1999). Dermal route exposure to mycotoxins can be mainly via handling contaminated material in mycotoxins research and in food industries. Parental exposure is simply via mother-to-child transmission through the placenta and after birth during breast feeding (Anyanwu, 2004). Exposure and diagnosis of mycotoxins should depend upon the absence of other readily diagnosed diseases and the discovery of a mycotoxin in suspected food. However, it is not sufficient to have isolated the fungus as one must be able to demonstrate the presence of biologically effective concentrations of the mycotoxin. Usually, mycotoxin exposure is mostly to be expected to occur in parts of the world where poor methods of food handling and storage are common, where malnutrition is a problem, and where few regulations exist to protect exposed populations. However, even in developed countries, specific subgroups may be vulnerable to mycotoxin exposure (Bennett and Klich, 2003).In south Africa in the Limpopo Province, for example, the populations in Mapate village consume more maize products than the rest of the population (Phoku *et al*., 2012) while, in the inner city populations are more likely to dwell in buildings that harbour high levels of mould (Barrett, 2000).

2.4.3 Mycotoxin detection

Recently mycotoxins are detected and quantified in food and feed using appropriate sophisticated and expensive laboratory equipment and skilled analytical chemists (Richard and Thurston, 1986; Cole, 1986; Beltran *et al*., 2009; Sparkman, 2000). Various applications of recent techniques have been described and applied to determination of mycotoxins (Ren *et al*., 2007; Ventura *et al*., 2006) in different fields such as food safety (Pozo *et al*., 2007), environmental analysis and academic research laboratories (Ibanez *et al*., 2008; Richardson, 2008). Furthermore, some screening techniques have been developed using antibody-based analysis such as Enzyme Linked ImmunoSorbent Assay (ELISA) and immunoaffinity column (IAC), (Ueno *et al*., 1991; Yu *et al*., 1998). Enzyme Linked immunoSorbent assay kits are commercially available for aflatoxins, cyclopiazonic acid, deoxynivalenol, fumonisins, ochratoxins, and zearalenone. This is usually a competitive assay in which the mycotoxin of interest from a sample competes with a labelled mycotoxin for a limited number of specific antibody-binding sites. Since the assay is competitive, the presence of the toxin is usually measured by the absence of colour (Xu *et al*., 2005a; Beltran *et al*., 2009). In addition, chromatographic techniques have been used and evolved from the beginning of mycotoxin

research and are still used for detecting, confirming and quantifying the presence of mycotoxins. Generally, chromatographic techniques used include thin layer chromatography (TLC), liquid performance liquid chromatography (HPLC) and gas chromatography/mass spectrometry (GC/MS) for accurate quantification of the compound detected (Sforza *et al*., 2006; Zollner and Mayer-Helm., 2006). Techniques for detecting and quantification of mycotoxins are summarized in Table 2.2. A number of detection methods such as fluorescence, ultraviolet absorption and including others have been combined with chromatographic methods (Hesseltine and Shotwell, 1973).

Table 2.2 Techniques for detection and quantification of mycotoxins, (Spanjer *et al*., 2008)

Techniques	Mycotoxins
TLC, ELISA, HPLC/ MS/MS, IAC, LC/MS/MS,	Aflatoxins
TLC, ELISA, HPLC, LC/MS	Citrinin
TLC, ELISA, HPLC, LC/MS/MS,	Cyclopiazonic acid
TLC, ELISA, HPLC, IAC, GC, LC/MS/MS,	Deoxynivalenol
TLC, ELISA, HPLC, IAC, LC/MS/MS,	Fumonisins
TLC, ELISA, HPLC, IAC, LC/MS/MS,	Ochratoxin
TLC, HPLC, LC/MS/MS,	Penicillic acid
TLC, HPLC, LC/MS/MS,	T-2 Toxin
TLC, ELISA, HPLC, IAC, LC/MS/MS,	Zearalenone

ELISA: Enzyme Linked ImmunoSorbent assay, AIC: Immunoaffinity column, GC/MS: Gas chromatography/mass spectrometry, HPLC: Liquid performance liquid chromatography, TLC: Thin layer chromatography.

High performance liquid chromatography and GC/MS have become the most widely used techniques for mycotoxin detection in food safety laboratories (Fiori *et al*., 2008; Shephard, 2008b). Moreover, HPLC separates a mixture of compounds on a stationary column using a carrier solvent such as methanol or acetonitrile, and the mycotoxins are detected and quantified in the sample as they pass through a specific detector. Gas chromatography/mass spectrometry separates a mixture of compounds on a stationary column using a carrier gas such as helium, and the mycotoxins are detected and quantified using a mass spectrometer. However, HPLC has become the most preferred technique for mycotoxins analysis, as it does not require the

derivatization step as typically applied in GC-based techniques. (Songsermsakul and Razzazi-Fazeli, 2008).

Ventura *et al*. (2006) developed an ultra-high pressure liquid chromatography/tandem mass spectrometry (UHPLC/MS/MS) method for the analysis of aflatoxins and ochratoxin A in beer. This very same technique was applied for the quantification of 17 mycotoxin contaminants in corn feeds and peanut butter, following a solid-phase extraction (SPE) for sample purification step (Ren *et al*., 2007). High performance liquid chromatography when coupled to mass spectrometry (MS), preferably to tandem mass spectrometry (MS/MS), which is ultra-high pressure liquid chromatography (UHPLC) presents the benefits of shorter run times, and narrower chromatographic peaks, which result in increased sensitivity and improved peak resolution (Pozo *et al*., 2007; Ren *et al*., 2007). Mass spectrometry works by ionizing chemical compounds to generate charged molecules or molecule fragments and measuring their mass-to-charge ratios (Sparkman, 2000). Major mycotoxins of great concern associated with harmful diseases in varying degrees to humans and animals, based on their occurrence will be discussed.

2.5 MAJOR MYCOTOXINS

2.5.1 Aflatoxins

Aflatoxin is an umbrella term referred to the group of mycotoxins that has in actual fact generated an interest in mycotoxin research in the world; since they were discovered in the 1960s with the outbreak of Turkey X disease, after the death of thousands turkey poults due to consumption of mould-contaminated peanut meal (Bennett and Klich, 2003; Dabrowski and Sikorski, 2005; Richard, 2008). There are mainly six different types of aflatoxins of concern produced and their chemical structures in (Fig. 2.8), which are aflatoxin B_1 (AFB_1), aflatoxin B_2 (AFB_2), aflatoxin G_1 (AFG_1), aflatoxin G_2 (AFG_2), aflatoxin M_1 (AFM_1) and aflatoxin M_2 (AFM_2) (Yin *et al*., 2008). The discovery of Turkey X disease has led to a growing awareness of the potential hazards of these substances causing diseases to humans and animals. In addition, it was soon discovered that the outbreak was not limited to turkeys, but to ducklings, chickens and young pheasants where heavy mortality rate was also experienced (Leeson *et al*., 1995; Diaz *et al*., 2008). The toxic substance was called aflatoxin produced by species *Aspergillus*, which are unavoidable contaminants and can grow on various foods at favourable conditions of

temperature and humidity (Martins *et al*., 2001). With respect to aflatoxigenic strains, there are great qualitative and quantitative differences in the toxigenic abilities displayed by the strains within each aflatoxigenic species. A typical example is where about half of the strains of *A. flavus* isolated from various substrates produced aflatoxins (Klich and Pitt, 1988), with concentrations of up to 100 000 micrograms per kilogram (µg/kg) (Cotty *et al*., 1994).

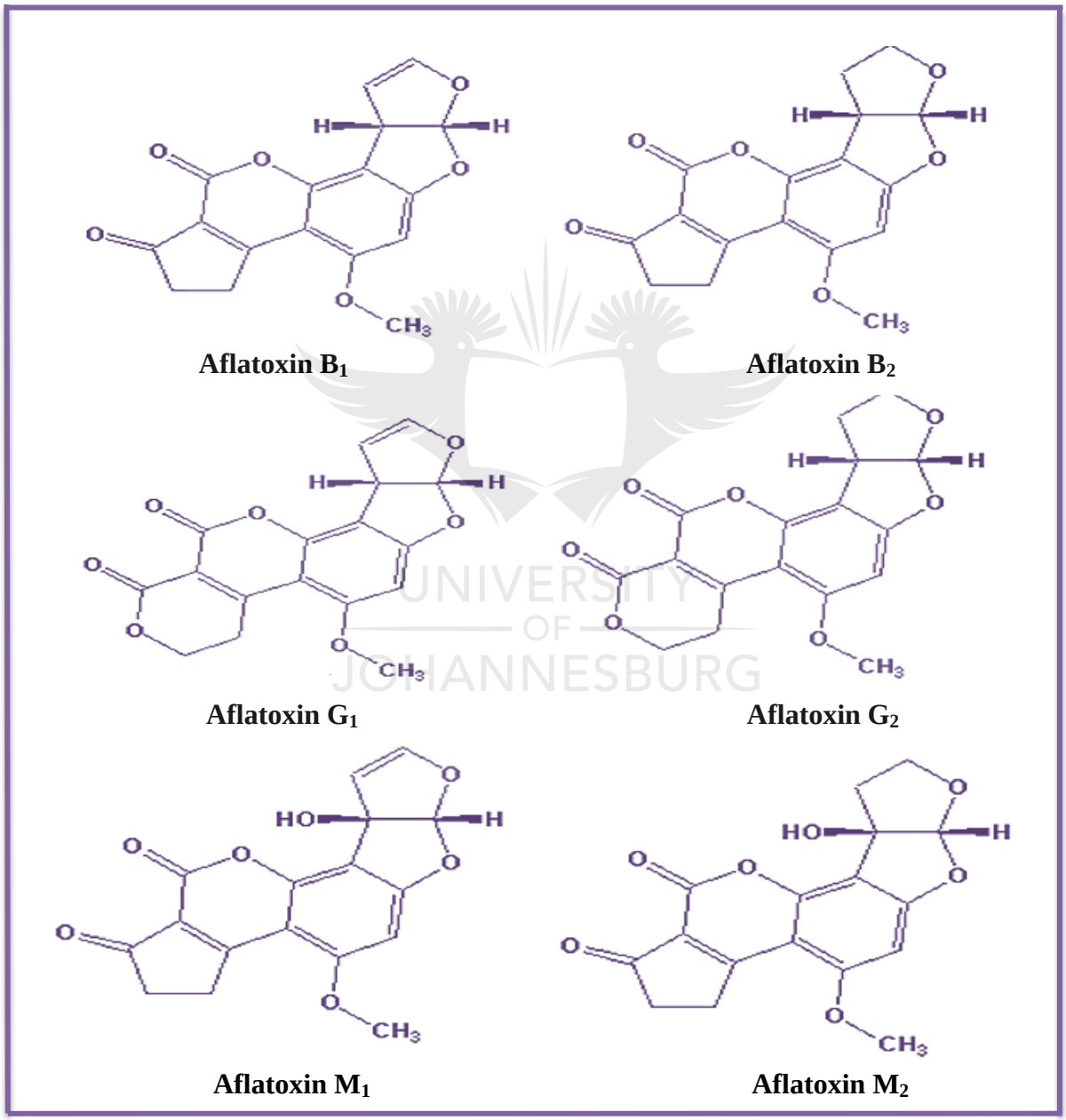


Figure 2.9 Chemical structure of aflatoxin B_1, aflatoxin B_2, aflatoxin G_1, aflatoxin G_2, aflatoxin M_1 and aflatoxin M_2, (Squire, 1981).

Many commodities support growth and aflatoxin production by aflatoxigenic fungi. Natural contamination commonly occur in corn, nuts and nuts products, oil and cotton seeds, spices and so forth. Sometimes crops become contaminated with aflatoxin in the field particularly before harvest, where it is usually associated with drought stress, insect activity, a poor timing of harvest, heavy rains during and after harvest, and an inadequate drying of the crop before storage (Sinha and Bhatnagar, 1998; Klich, 2002b; Cotty and Jaime-Garcia, 2007; Paterson and Lima, 2010). Although aflatoxins are not automatically produced whenever grain becomes mouldy, the risk of aflatoxin contamination is greater in damaged, mouldy corn than in corn with little mould. However, contamination may also persist in storage depending on the moisture content and the humidity of the surroundings (Richard, 2007; Wilson and Payne, 1994). Generally, maize is the most common commodity of greatest concern worldwide, since it is dietary staple, which serves as an additional food and nutritional supplement for both animals and humans in most countries and is usually grown under climate conditions, which favours fungal infestation and aflatoxin production (CAST, 2003; Nithiyaa *et al*., 2012). Diet is the major way through which humans as well as animals are exposed to aflatoxins. Another route of exposure to aflatoxin can be through ingestion of contaminated milk containing aflatoxin M_1 from animals which is derived from consumed AFB_1 (Gimeno, 2004; Wild and Gong, 2010). Furthermore, occupational exposure to aflatoxins in agricultural personnel, those working in oil mills, and granaries has been demonstrated (Sorenson *et al*., 1984).

Of the six different types of aflatoxins, B and G-toxins refer to the blue and green fluorescent colours produced by these compounds under ultra-violet (UV) light on TLC plates, while the subscript numbers 1 and 2 indicate major and minor compounds (Pitt, 2000a).The M-toxins are oxidative metabolic products of AFB_1 and AFB_2, have been identified from milk of lactating cows consuming AFB_1 contaminated feeds from animals (Yoder, 1980; Peraica *et al*., 1999a). Aflatoxin B_1, the principle member of the group, has caused a number of deaths of poultry and other domestic animals. If an animal such as a dairy cow ingest AFB_1 in the diet, AFB_1 is rapidly absorbed in the gastrointestinal tract and is transformed into the metabolite AFM_1, which appears in the blood after 15 mins and is secreted in the milk (van Egmond, 1989; Battacone *et al*., 2003). Because AFM_1 is stable in raw milk and processed milk products and is relatively resistant to pasteurization, it is retained in pasteurized milk, powdered milk and infant formula

(Yousef and Marth, 1989; Galvano *et al*., 1996). Additionally, the concentration of AFM_1 found in milk depends on various factors which include animal breed, lactation period, mammary infections and so forth. In the study conducted by van Egmond and Dragacci (2001), data demonstrated that up to 6% the amount AFB_1 ingested is secreted into the milk as AFM_1. Similarly to maize, milk and milk products are consumed on daily basis, and besides the fact that they are of primary importance to children, most countries have set acceptance maximum limit of AFB_1 50 nanogram per kilogram (ng/kg) in feed (EC, 2003) and of AFM_1 (500 ng/L) in milk established by U.S Food and Drug Administration (U.S. FDA, 2011).

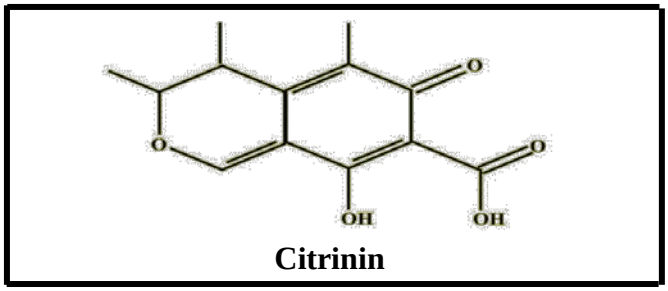
Aflatoxin is associated with toxicity and carcinogenicity in human and animal populations from many parts of world (Shank *et al*., 1972; Peers and Linsell, 1973; IARC, 1993a). The diseases caused by aflatoxin referred to as aflatoxicosis, is poisoning that result from ingestion of aflatoxins in contaminated food and feed. Thus, acute aflatoxicosis leads to death whereas chronic aflatoxicosis leads to cancer, immune suppression and other delayed pathological conditions (Hsieh, 1988). The liver is the main organ targeted, with liver damage resulting when AFB_1 is fed to domestic and non-domestic animals like cattle, horses, rabbits, and other non-human primates (Cullen and Newberne, 1994; Eaton and Groopman, 1994; Miller, 1992). After absorption from the gastrointestinal tract, the aflatoxins are transported by the portal circulation and are filtered from the blood by the liver (Miller and Wilson, 1994). The hepatotoxicity and carcinogenicity effects of AFB_1 have been classified as a group 1 human carcinogen by the International Agency on Research on Cancer (IARC, 2002). Initially, the IARC classified AFM_1 as a possible carcinogen for humans (group 2B) due to limited toxicological data (IARC, 1993a). The genotoxicity and carcinogenicity of AFM_1 have been reported *in vivo*, although lower than those of AFB_1, and its cytotoxicity effects definitively demonstrated (Caloni *et al*., 2006). However, due to sufficient evidence in humans for the carcinogenicity of naturally occurring mixtures of aflatoxins, AFM_1 moved from group 2B to group 1 human carcinogen (IARC, 2002).

In recent years, several outbreaks of acute aflatoxicosis in Africa have occurred mostly among adults in rural populations where large doses of aflatoxin resulting from consumption of contaminated maize (Peraica *et al*., 1999a; Strosnider *et al*., 2006). In India, an outbreak of hepatitis affected 400 people, of whom 100 people lost their lives due to the consumption of

maize that was heavily contaminated with aflatoxin at 15 milligram per kilogram (mg/kg). Furthermore, consumption of toxin by some of the affected adults was 2 to 6 mg of aflatoxin in a single day (Krishnamachari *et al*., 1975). It was observed that the acute lethal dose for adult humans were about 10 to 20 mg of aflatoxins (Pitt, 2000a). A case of suicide attempt was reported in 1966, to have occurred in the United States of America (USA) a woman who had ingested over 40.5 mg of purified AFB_1. Following exposure she was observed with symptoms of non-pruritic, headache, nausea, and macular rash. A multiple examination of her urine and blood, and X-ray, ultrasound, and computerized axial tomography analyses of her abdomen, liver, and spleen appeared normal. In addition, these findings suggest that the hepatotoxicity of AFB_1 may be lower for in well- nourished individuals than in experimental animals (Willis *et al*., 1980). Currently, a vast majority of people worldwide are at risk of chronic exposure to aflatoxin in food. Peanuts are one of the main sources of human exposure to aflatoxin; hence, it is one of the most susceptible crops to aflatoxin contamination (Liu and Wu, 2010). The occurrence of liver cancer differs widely from country to country, but it is in China, Philippines, Thailand, and many African countries (Bennett and Klich, 2003). In developed countries, sufficient amounts of food combined with regulations that monitor aflatoxin levels in food protect human populations from significant aflatoxin ingestion. However, in countries where populations are facing malnutrition, where regulations are both not enforced and non-existent, repetitive ingestion of aflatoxin contaminated foods and feeds with high levels are likely to occur (Cotty *et al*., 1994).

2.5.2 Citrinin

The mycotoxin citrinin (Fig. 2.9) is a yellow compound (Altug, 2003) produced by several species of the genera *Aspergillus*, *Penicillium* and *Monascus* species (Kim *et al*., 2007; Abramson *et al*., 2009).



Citrinin

Figure 2.10 Chemical structure of Citrinin, (Iwahashi *et al*., 2007).

Penicillium citrinum, a major producer of citrinin (CTN) is widely distributed in all rice producing parts of the world. It has been isolated from rice produced in Asian countries (Fink-Gremmels *et al*., 1991). Contamination of CTN producing mould species were reported in various agricultural commodities after harvest particularly in stored cereal grains such as wheat, barley, oats, rice, corn beans, fruits, fruit and vegetable juices, spices and herbs (CAST, 2003; Meister, 2004; Flajs and Peraica, 2009; EFSA, 2012a). Growth on stored rice, especially polished rice is usually accompanied by formation of the pigment that causes the surface of rice kernels to appear to yellow (Wong and Koehler, 1981). Barley has been reported to be a good substrate for the growth of many toxigenic fungi capable of producing citrinin along other cereals employed for producing beer, (Galvano *et al*., 2005). Citrinin is likely to be destroyed during brewing and studies have shown that over 90% is destroyed during germination of barley, with no citrinin surviving the mashing process of making wort from malt (Krogh *et al*., 1974). Presence of propionic acid destroys citrinin when added as a preservative to protect stored barley destined for animal feed from moulding during storage. Possibly the major characteristics of its occurrence is that it often co-occurs with ochratoxin A in cereals and most isolates of fungi that produce citrinin also produce ochratoxin A, although citrinin has been reported much less frequently (EFSA, 2012a). According to Wong and Koehler, (1981) CTN was investigated as an antibiotic, based on its antibacterial effects. Furthermore, CTN has been associated with an acute form of cardiac beriberi referred to as yellow rice disease in Japan, when isolates of *P. citrinum* produced high levels of CTN (Saito *et al*., 1971).

Citrinin is a strong kidney toxin that has been identified as contaminants of yellow peanut kernels from damaged pods. However, toxicity of citrinin is low when compared to its co-occurring toxin, ochratoxin A. Citrinin, a mycotoxin that is often found as a natural contaminant in foods used by humans and animals has been demonstrated to have cytotoxic and genotoxic effects, but its acute toxicity varies (Bennett and Klich, 2003). Some toxicity studies have showed that citrinin acted in as a nephrotoxin, damaged the proximal tubules of the kidney, and was implicated as a potential causative agent Balkan endemic nephropathy (BEN) associated with an increased frequency of urinary tract tumours in animals and humans (IARC, 1986; Zaied *et al*., 2012). Nevertheless, it would seem unlikely in normal circumstances that citrinin presents much risk to humans as it is unstable in cereal processing so that the greatest risk is probably to

livestock, particularly pigs, feeding on contaminated cereal products (Xu *et al*., 2006). On the other hand, information available on the toxic effects of citrinin in animals show its nephrotoxic nature as well as teratogenic effects in rabbits, poultry, dogs, and rats and mice along with induction of apoptosis (Chan, 2007; Kumar *et al*., 2007; Singh *et al*., 2008). In biological research CTN is used as a reagent. It induces mitochondrial permeability pore opening and inhibits respiration by interfering with complex I of the respiratory chain. Citrinin can permeate through the human skin. Although no significant health risk is expected after dermal contact in agricultural or residential environments, dermal exposure should nevertheless be limited (Boonen *et al*., 2012). A study conducted by Stoev and his colleagues it was demonstrated that the presence of CTN in animal feed from South Africa was high (Stoev *et al*., 2010b), while high incidence rates of CTN producing species were found to occur in maize samples from Cameroon (Ngoko *et al*., 2001). Additionally, CTN content of 0.28-2458.80 μg/kg was found in commercial red fermented rice products, of which lipid extracts range from 1.8- 4.7 ng/kg caused 50% cell death to human embryonic kidney cell (HEK) (Li, *et al*., 2003; Liu, *et al*., 2005).

2.5.3 Fumonisins

Fumonisins are a group of characterized toxins produced by a limited number of *Fusarium* moulds, of which *F. verticillioides* and *F. proliferatum* are the most important producers, as they frequently contaminate agricultural commodities throughout the world especially in cool and humid climates (Dutton, 1996; Sewram *et al*., 1999; Marasas, 2001; Vanara *et al*., 2009).Traditionally, *F. verticillioides* is the fungal species associated with fumonisin production (Marasas *et al*., 1988; Seifert *et al*., 2003). Maize is the most commonly contaminated crop, and fumonisins are the most common mycotoxins in maize, although these toxins can also occur in a few other crops (Patel *et al*., 1997; D'Mello, 2003). The contamination of foods and feeds with *F. verticillioides* used for humans and livestocks generally reflects the degree of fungal infection of the original crops during a particular season, the occurrence of which is influenced by a number of factors such as the origin, drought stress and insect damage (Placinta *et al*., 1999; Turner *et al*., 1999; Soriano and Dragacci, 2004). However, the levels of contamination varies considerably in different regions, ranging from negligible to more than 100 parts per million (ppm). Although production of the toxin generally occurs in the field, continued production of

toxin during post-harvest storage if proper conditions are not maintained also contributes to the overall levels (Mubatanhema *et al*., 1999; Chu, 2001; 2002; Ezekiel *et al*., 2008).

Of the known fumonisins there are three that occur together, named fumonisin B_1 (FB_1), fumonisin B_2 (FB_2) and fumonisin B_3 (FB_3). Chemically, FB_1 is a derivative (diester) of propane-1,2,3-tricarboxylic acid of 2-amino-12,16-dimethyl-3,5,10,14,15-pentahydroxyicose (Fig. 2.11) (Gelderblom *et al*., 1988; 1992a, b; Shier, 1992; Nelson *et al*., 1993; Scott, 1993; Marasas, 1995; Riley *et al*., 1996).

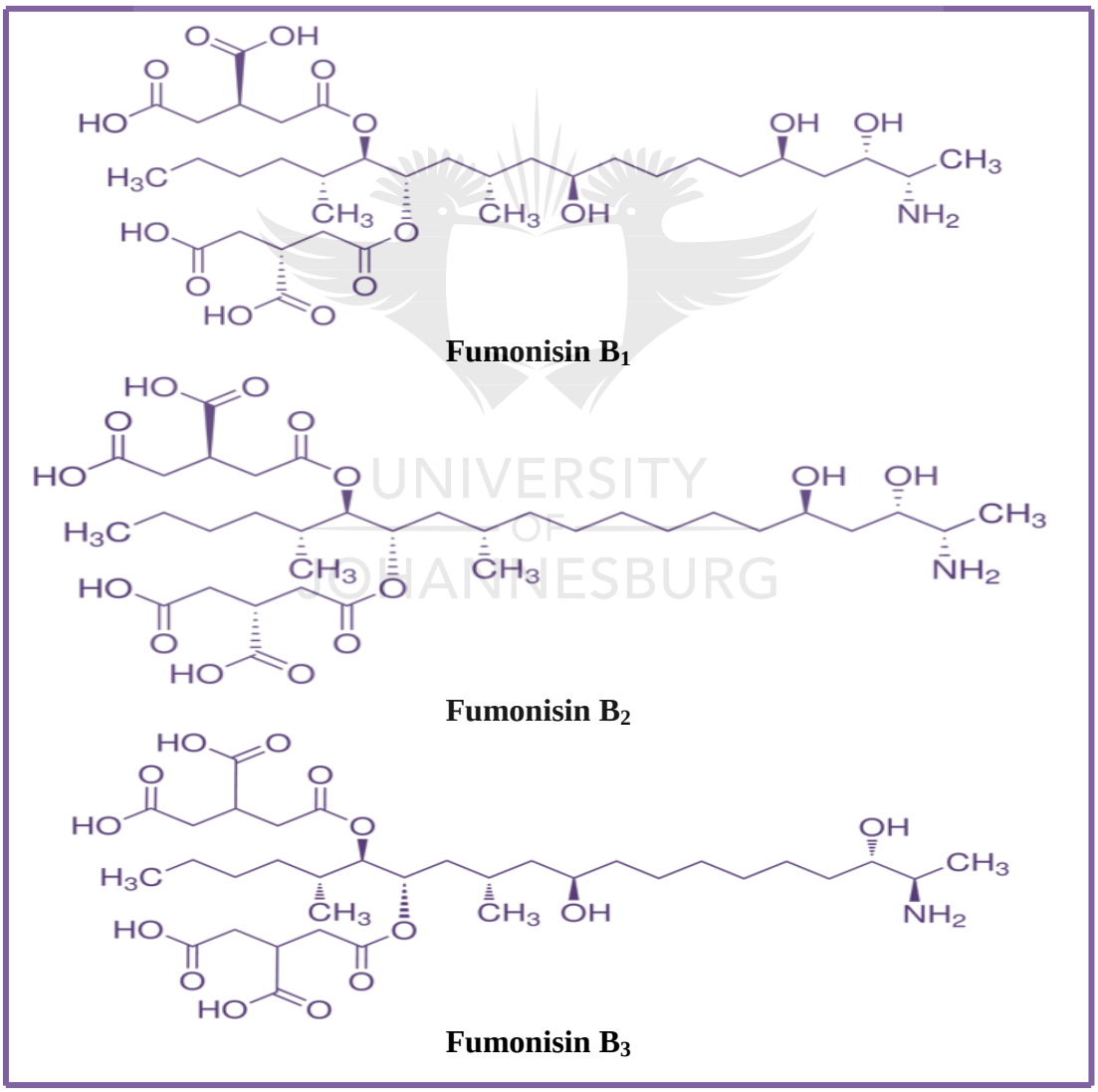


Figure 2.11 Chemical structures of fumonisin B_1, fumonisin B_2 and fumonisin B_3, (Marasas *et al*., 2004).

Fumonisin B_1 is always the most abundant and of economically and toxicologically significance, followed by FB_2 and FB_3 which are less toxic and occur in very low concentrations (Peraica *et al*., 1999a). These mycotoxins cause several losses in the quality of the maize and have been recorded to produce a wide range of biological effects to the health of consumers, both humans and animals (Zinedine and Manes, 2009). In addition, higher contamination levels of FBs especially FB_1 in specific products such as maize and maize-based products have been well established in many studies (Stockmann-Juvalla and Savolainen, 2008). The major health concerns linked with fumonisins can be acute toxic effects, including a fatal disease known as equine leukoencephalomalacia (ELEM) in horses, pulmonary oedema in pigs, nephrotoxicity and liver cancer in rats, the latter being a more chronic conditions Symptoms of ELEM include drowsiness, blindness, staggering, and liquefaction of brain tissue, reduced feed intake and weight gain, and in pigs, hydrothorax in which the animals' lungs are filled with fluid (Colvin and Harrison, 1992; Gelderblom *et al*., 1992a; Munkvold *et al*., 1998; Pitt, 2000a; Pascale *et al*., 2002).

The WHO's International Programme on Chemical Safety (IPCS) and the Scientific Committee on Food (SCF) of the European Commission evaluated the risks of fumonisin B_1. The tolerable daily intake (TDI) for FB_1, FB_2, FB_3, was determined alone or in combination of 2 µg/kg body weight in humans (SCF, 2000a; WHO Technical Report Series, 2002). To date, no data on the kinetics and metabolism of FB_1 in humans have been reported. Much research on animals has been conducted, but then again it might not be comparable to humans. Moreover, in rats the elimination of FB_1 is very rapid, but in humans it could be much slower taking into consideration the body weight and prolonged chronic exposure (Gelderblom *et al*., 1993; Stockmann-Juvalla and Savolainen, 2008; Dutton, 2009). There are more than a few possible pathways that cause toxic effects of FB_1. Most toxic effects are due to altered sphingolipid metabolism by inhibition of ceramide synthase. Production of reactive oxygen species (ROS) may occur. However, this increase oxidative stress and induce lipid peroxidation could cause cell damage. Studies have showed decreased levels of glutathione (GSH) in liver, but other studies showed even elevated levels (Wang *et al*., 1991; Norred *et al*., 1992; Merrill *et al*., 1993; Yoo *et al*., 1996; Merrill *et al*., 1997; Riley, 1998; Abnet *et al*., 2001).

Although their effects on humans are difficult to determine, consumption of fumonisin-contaminated maize have been statistically associated with higher rates of oesophageal cancer; idiopathic congestive cardiopathy and eclampsia (Rheeder *et al*., 1992; Dutton, 2009) and neural tube birth defects (Marasas *et al*., 2004; Missmer *et al*., 2006). Neural tube defects (NTDs) are referred to defects of the brain and spinal cord in the embryo resulting from failure of the neural tube to close (McComb and Chen, 1996). Epidemiological studies have indicated insufficiency of folate as a major risk factor for NTD (Blom *et al*., 2006). Additionally, FB_1 disrupts sphingolipid metabolism and hence this could affect folate uptake and cause NTD (Stockmann-Juvalla and Savolainen, 2008). Along the Texas-Mexico border in 1990 and 1991, a sudden outbreak of NTDs occurred. This outbreak was linked to intake of high levels of FB_1 that were observed in maize during previous years (Missmer *et al*., 2006). In agreement to this, also regions in China and South Africa with high maize consumption show a high prevalence of NTDs (Cornell *et al*., 1975-1980; Gelineau-van Waes *et al*., 2005; Missmer *et al*., 2006). It is assumed that there is a connection between the occurrence of *F. verticillioides* and human oesophageal cancer. A low socio-economic status and a less variated diet that mainly consists of maize and maize-based products are associated with the appearance of oesophageal cancer. These assumptions are from epidemiologic studies in various countries. Research studies have shown that a higher level of concentrations of FB_1, FB_2 and *F. verticillioides* are present in maize growing in regions with a high percentage of oesophageal cancer (Wild and Gong, 2010). As a result, people with a high maize intake are at higher risk to develop oesophageal cancer than people with low maize intake. This is however, observed in regions such as in Italy, Iran, Kenya, Zimbabwe, United States and Brazil with high incidence of oesophageal cancer (Yoshizawa *et al*., 1994; Dombrink-Kurtzman and Dvorak, 1999; IPCS, 2001). Another study on the relationship between sphingolipid levels and the incidence of cancer didn't show any significant relationship between serum sphingolipids and risk of oesophageal cancer. This is, to a certain extent remarkable, because elevated levels of sphingolipids sphinganine and sphingosine are believed to be biomarkers for exposure of FB_1 (Stockmann-Juvalla and Savolainen, 2008). Since contamination on grain cereal has been proven to be one of the major causes of toxicity affecting animals and human, special attention must therefore be paid during the processes of storage, handling and packaging.

2.5.4 Ochratoxins

Ochratoxin is a mycotoxin that comes in three secondary metabolite forms, ochratoxin A (OTA), ochratoxin B (OTB), and ochratoxin C (OTC). All are produced by *Penicillium* and *Aspergillus* strains. The three forms (Fig. 2.12) differ in that OTB is a non-chlorinated form of OTA and that OTC is an ethyl ester form OTA (Bayman and Baker, 2006). The most frequent occurring one is ochratoxin A, which is the most toxic form of ochratoxin. This occurs as a colourless crystal at room temperature under normal light and exhibits green and blue fluorescence under UV. Ochratoxin B and OTC have been found to occur naturally at low frequency (IARC, 1976; Speijers and van Egmond, 1993; O'Brien and Dietrich, 2005).

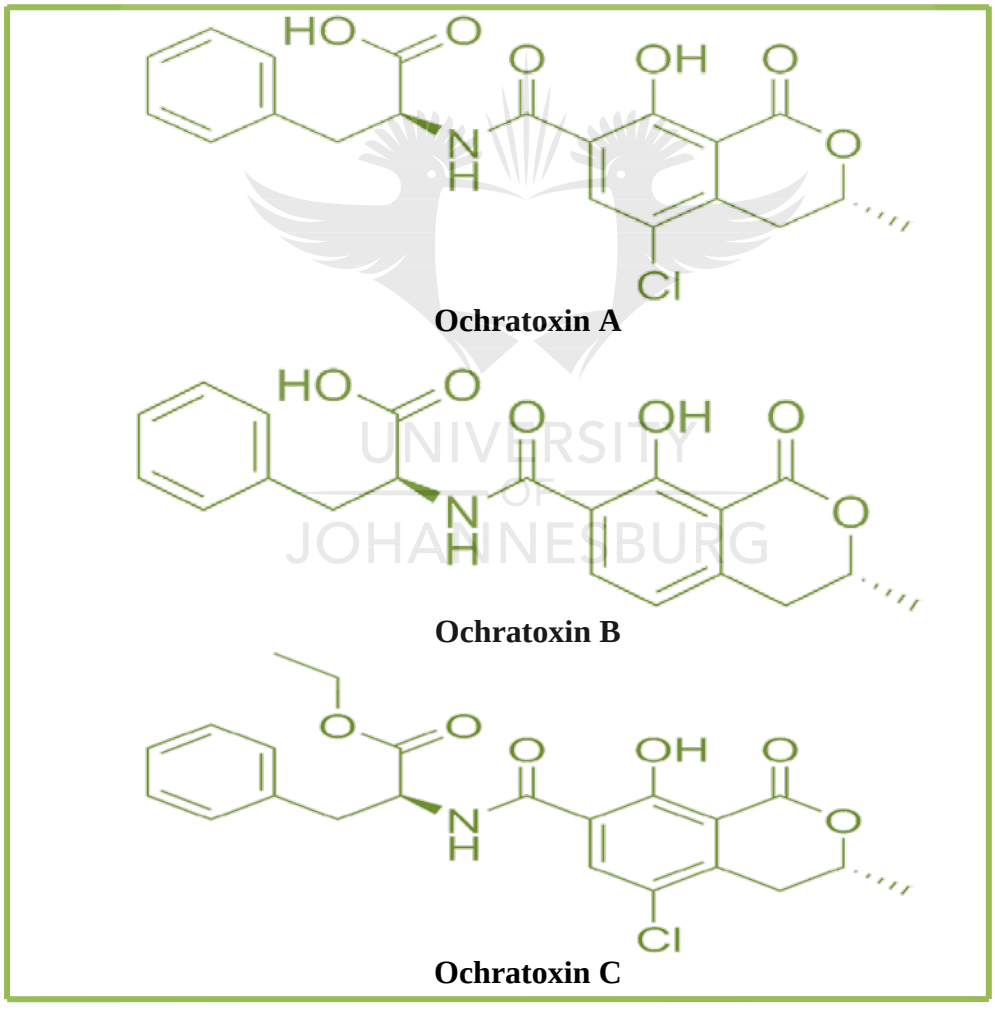


Figure 2.12 Chemical structures of ochratoxin A, ochratoxin B and ochratoxin C, (Mally *et al*., 2005).

Ochratoxin contaminates maize, pork, barley, coffee, beans, wheat, rice, grapes, oats and peanuts as well as on all kinds of food commodities of animal origin in many countries (De Vries *et al*., 2002; Samson *et al*., 2002). Ochratoxin contamination has been shown to occur both during pre-harvest and post-harvest, with post-harvest production usually regarded as most predominant in food and feeds (Pittet, 1998). Ochratoxin A was first isolated in mid 1960s in South Africa during laboratory studies in search for new toxic metabolites from *A. ochraceus* (Van der Merwe *et al*., 1965) and was later shown as a secondary metabolite of *Penicillium* species in temperate climates.

Ochratoxin A the main toxin of this group shows biological and biochemical effects such as; teratogenicity, nephropathy, enhancement of lipid peroxidation, partial inhibition of ATP dependent calcium uptake, carcinogenicity and inhibition of cell-mediated immune response (Kuiper and Scott, 1989; StÆrmer, 1992; Wu *et al*., 2011). Ochratoxin A is assumed to cause Balkan endemic nephropathy (BEN), a chronic kidney disease in humans when it is digested in combination with FB_1 and citrinin and also causes urothelial tumours, both endemic to Balkan states (Radonic and Radosevic, 1992; Radic *et al*., 1997). Ochratoxin A, CIT, and FB_1 are known to be nephrotoxic mycotoxins and are reported in cases of nephropathy in several countries (Voss *et al*., 2001; Stoev, 2008). Natural nephropathy was observed in South African pigs involving several mycotoxins such as OTA, PA and FB_1. Furthermore, the study demonstrated that simultaneous exposure to the mentioned mycotoxins might be of significant importance and could be crucial for development of chronic renal failure observed in BEN, especially after long-term consumption of the same mycotoxins (Stoev *et al*., 2010a). Balkan endemic nephropathy is a chronic tubulointerstitial nephritis that was reported affecting residents of rural populations in Bosnia, Bulgaria, Croatia, Romania, and Serbia (Petkova-Bocharova *et al*., 1991; Plestina, 1992; Djukanovic and Radovanovic, 2003). Although BEN was first described in the literature in the late 1950s, anecdotal reports suggest that this fatal disease was present in the above mentioned countries many decades before (Bukvic, 2006). Human exposure can occur directly by consumption of contaminated food derived from plant and indirectly by consumption of animal tissues from livestock exposed to contaminated feed (Petkova *et al*., 1988). Additionally, since OTA is fat soluble and not readily excreted, it accumulates in the depot fat of affected animals, and therefore is ingested by humans eating meat. Another source is bread made from wheat

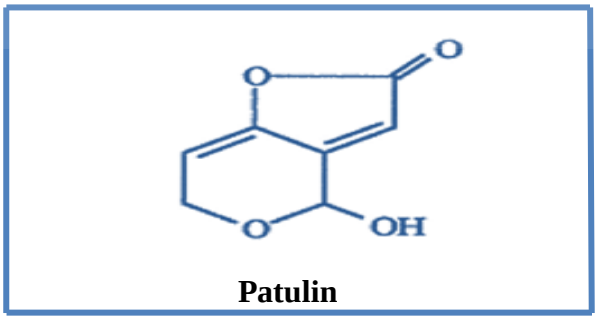
containing the toxin (Pitt, 2000a). Nevertheless, there is no acute phase of the disease; signs and symptoms include fatigue, headache, abdominal pain, loss of body weight and pale skin (Heptinstall, 1974; Radonic and Radosevic, 1992; Vukelic *et al*., 1992).

Ochratoxin A has been reported frequently in higher concentrations in human blood and in human milk from endemic regions than control regions (Castegnaro *et al*., 1991; Bretholtz *et al*., 1993; Radic *et al*., 1997; Petkova et al., 1991; Peraica *et al*., 1999b). Ochratoxin A has been measured in the blood and urine of exposed individuals around the world. The concentrations are exceptionally high in Bulgaria, where endemic BEN occurs. Concentrations of ochratoxin A in the blood have been measured at up to 100 μg/kg in Bulgaria (Clark and Snedeker, 2006) and up to 66.2 μg/kg in Tunisia (Abid *et al*., 2003). Urinary concentrations have been measured at up to 148 μg/kg for girls in Sierra Leone (Jonsyn-Ellis, 2000) and 0.604 μg/kg for individuals with BEN in Bulgaria (Castegnaro *et al*., 1991). The highest concentration in breast milk was 1,890 ng/kg in Egypt (Hassan *et al*., 2006). In another study in Tunisia, OTA has been detected in high concentrations in the blood and food of patients with kidney impairment of unknown aetiology (Maaroufi *et al*., 1995a, b). Although at low concentrations, compared to endemic regions, OTA has been found in African crops intended for animal feed (Stoev *et al*., 2009). Despite intensive research at both the epidemiological and the experimental levels, the mode of action of OTA and its causal association to BEN and urothelial tumours remain unclear (Kuiper *et al*., 1993; Studer, 1995; Studer *et al*., 2000). Total daily intake of ochratoxin A vary considerably among countries, depending on, amongst other influences, food-handling methods, and has been estimated based on total diets or on consumption of specific contaminated foods or beverages. The highest estimated daily intake was 1.21 μg for adults with BEN in Bulgaria (Clark and Snedeker, 2006), and the highest estimated daily intake for children was 3.6 ng/kg of body weight for Swiss children who consumed grape juice. Ochratoxin A has been detected in fresh grapes, grape juice, dried vine fruits and all types of wine throughout the world. It was found in Cabernet Sauvignon grapes from Portugal at a concentration of 115.6 μg/kg (Serra *et al*., 2006), in grape juice at 0.337 μg/kg (Clark and Snedeker, 2006), and in dried fruit (raisins, currants, and sultanas) purchased in the United Kingdom at concentrations of up to 53.6 μg/kg (Rizzo *et al*., 2002).The IARC has classified OTA as a compound possible for human carcinogen (Group 2B) based on the sufficient evidence of carcinogenicity in experimental animal studies (IARC, 1994).

2.5.5 Patulin

Patulin (4-hydroxy-4H-furo[3,2-c]pyran-2(6H)-one) (Fuchs *et al*., 2008) is a mycotoxin that forms the smallest group of toxic metabolites referred to as polyketides, and is reported to be produced by a variety of fungal species, in particular, *Aspergillus*, *Byssochlamys* and *Penicillium* species (Moss 2008; Chun *et al*., 2009). Patulin (PAT) has been found to occur in a number of foods including apples juice, apples, pears, avocados, grapes, tomatoes, mangoes and in moulded grains (Pitt and Hocking, 1997c). For humans, the major potential dietary sources of PAT are apples and apple juices made from affected fruit. Patulin is not considered a potent toxin, however a number of studies have shown that it is genotoxic, which has led to some theories that claim that it may be a carcinogen, though animal studies have remained questionable (Hopmans, 1997).

Furthermore, in some countries such as Europe and New Zealand, PAT as structurally outlined in (Fig. 2.13), is considered a "possible toxin" and regarded as the most hazardous mycotoxin in fruits, particularly apples, pears, and their products (Murillo *et al*., 2009). In general, fungal growth and subsequent production of PAT usually occurs only where the surface tissue of fruit has been damaged which increases the probability of fungal contamination, mainly by *P. expansum*, commonly identified as the "blue mould rot" appears to be the fungi normally responsible for patulin in juice. Surface damage in most cases, may be caused by insects, storm damage, handling procedures and poor storage conditions that promote bruising and rotting (Rychlik and Schieberle, 2001; Sewram *et al*., 2000; Altug, 2003; Ritieni, 2003).



Patulin

Figure 2.13 Chemical structure of Patulin, (Bjarnsholt and Givskov, 2007).

Although PAT was studied initially as a potential compound possessing a broad spectrum antibiotic properties, research studies have demonstrated the toxicological properties as

mutagenic, immunotoxic, neurotoxic causing adverse health effects on the gastrointestinal tissue (Wichmann *et al*., 2002) and to exhibit adverse effects on the developing foetus in rats (Hopkins, 1993; Smith *et al*., 1993). It has also been found to induce oxidative stress response in mammalian cells, generate reactive oxygen species, and induce apoptosis in human leukaemia cells (HL-60) (Wu *et al*., 2008). In addition, there is a concern that similar adverse effects may occur in humans through long-term consumption of PAT-contaminated foods and beverages. However, the IARC has classified PAT as category 3, not classifiable based on its carcinogenicity in humans (IARC, 1993b). A provisional maximum tolerable daily PAT intake (PMTDI) have been estimated to be 0.2 μg/kg bw/day for children and 0.1 μg/kg bw/day for adults, well below the tolerable intake established by JECFA (Wouters and Speijers, 1995; Bloger, 2002). In the EU, the permissible limit for PAT content has been set to 50 μg/kg for fruit juice, juice ingredients in other beverages, spirit drinks, ciders and other fermented drinks derived from apple and 25 μg/kg in solid apple products including apple compotes and apple puree, and 10 μg/kg for baby foods other than processed cereal-based foods for infants and young children (EC, 2006).

Furthermore, significant health effects of PAT raised attention and the need of several surveys on level of PAT contamination in some countries. A survey on 45 samples of apple juice concentrates analysed for PAT in Turkey found that 60% of samples were contaminated with concentrations ranging from 19.1 to 732.8 μg/kg and 40% of these samples had contamination levels higher than 50 μg/kg (Yurdun *et al*., 2001). Of 135 samples analysed in Italy, 47 samples were contaminated with levels ranging from 1.58 to 55.41 μg/kg (Spadaro *et al*., 2007). Results from the South African survey of 60 commercial apple products for PAT contamination showed that none of the samples had PAT higher above 50 μg/kg and the maximum level reported was 45 μg/kg (Loggott and Shephard, 2001).

2.5.6 Sterigmatocystin

Sterigmatocystin (STC) is a secondary metabolite (Fig. 2.14) structurally related to AFB_1 (Versilovskis *et al*., 2008). Sterigmatocystin in commonly produced by different fungal species such as *Aspergillus, Penicillium, Bipolaris, Chaetomium* and *Emericella* (Atalla *et al*., 2003; Versilovskis and De Saeger, 2010; Ranka *et al*., 2011) and is an important contaminant of water-

damaged building materials and dwellings (Gravesen *et al*., 1999; Nielsen *et al*., 1999; Engelhart *et al*., 2002). Sterigmatocystin was first isolated in brown rice stored in warehouses under natural conditions in Japan (Takahashi *et al*., 1984). It is a precursor of AFB_1 in the biological transformation, although it appears to occur less frequently and its acute and chronic toxicity is considerably lower than that of AFs (Barnes *et al*., 1994; Wilkinson *et al*., 2004). This toxin has been reported to contaminate various foods and feeds and to cause serious health and economic problems worldwide but only a limited number of surveys have been carried out (Jelinek *et al*., 1989; Versilovskis *et al*., 2008). The instances on natural sources of exposure reported have normally been on mouldy or poor quality materials such as maize, wheat, green coffee beans and cheese (Lund *et al*., 1995; Scudamore *et al*., 1996; Rao and George, 2000).

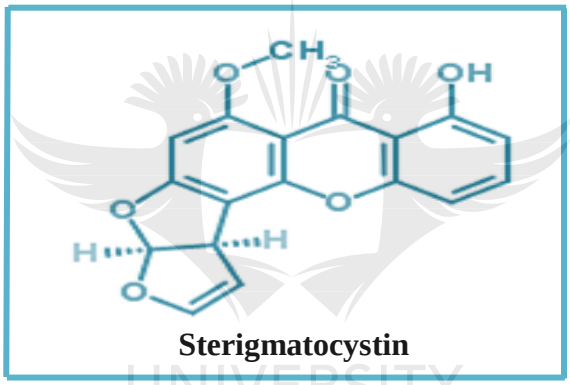


Figure 2.14 Chemical structure of Sterigmatocystin, (Versilovskis and De Saeger, 2010).

Sterigmatocystin adverse effects are much similar to those of AFB_1, thus it is considered as a potent carcinogen, mutagen, and teratogen that has been shown to affect various species of experimental animals, frequently observed in the liver and the lung (Tong-Xi *et al*., 2000; Kusunoki *et al*., 2011). Furthermore, some reports have associated STC and gastric cancer in China, although low vitamin and other dietary factors have also been implicated (Huang *et al*., 2004). According to Sum *et al*. (2002) STC may have some negative effects on human immuno-system and induce apoptosis of human peripheral blood lymphocytes (HPBLs) *in vitro*. There are no reports and epidemiological data referring directly to human toxicology. However, some evidence indicates that exposure to sterigmatocystin has a possible link to an increased risk of developing cancer in humans (Zhang *et al*., 1997; Sun *et al*., 1998). Many other reports have

associated sterigmatocystin to toxicity and mutagenicity (Sekijima *et al*., 1992; Sivakumar *et al*., 2001; Huang *et al*., 2002). Sterigmatocystin is classified as Group 2B with possible carcinogenicity in humans by the International Agency for Research on Cancer (IARC, 1987).

Although STC has exhibited some potential toxicity effects, there are no specific current regulations for recommended maximum limits in food and feed. However, some EU countries (Czech republic and Slovakia) have set relatively low maximum limits at 5 μg/kg for rice, vegetables, potatoes flour, poultry, meat, milk and 20 μg/kg for other foods, depending on the nature of the product (Stroka *et al*., 2004). Also, soon after STC was recognised as a highly toxic compound, the California Department of Health services used LD 50 (lethal dose) values from the cancer potency database to produce "no significant risk" intake levels for humans. The levels resulting were 8 μg/kg bw/day for a 70 kg adult EMAN (European Mycotoxin Awareness Network, 2007).

2.5.7 Trichothecenes

Trichothecenes (THs) are a very large family of chemically related metabolites produced essentially by a wide range of *Fusarium*, *Cephalosporium*, *Trichoderma*, *Trichothecium*, *Myrothecium*, *Stachybotrys*, and *Verticimonosporium* genera (Ueno, 1989; Torp and Nirenberg, 2004; Burkin *et al*., 2008; Mattila, 2010). The genus *Fusarium* typically develop during prolonged cool, wet growing and harvest seasons to produce FHB in cereal grains (Bottalico and Perrone, 1998; Goswami and Kistler, 2004; Desjardins, 2006; Mattila, 2010). The THs comprise a group of closely related compounds designated sesquiterpenoids having a tetracyclic 12, 13-epoxytrichothecene skeleton (CAST, 2003). In addition, there are four types of THs belonging to type A, B, C and D, of which the compounds belonging to type And B are the most important in food context. The type A compounds includes some of the most acutely toxic metabolites such as T-2 toxin, HT-2 toxin, diacetoxyscirpenol (DAS), monoacetoxyscirpenol and neosolaniol. The type B compounds include nivalenol (NIV), deoxynivalenol (DON), fusarenon X (FUS X) and diacetylnivalenol (Eriksen, 2003; Sudakin, 2003; Pestka and Smolinski, 2005). The most frequent THs of concern are DON, NIV, DAS and T-2 toxin (which is the most toxic) and whose chemical structures are shown in Fig. 2.15 (Yiannikouris and Jouany, 2002; Eriksen and Pettersson, 2004). These THs are of special concern due to their health effects on humans and

animals (Holt *et al*., 1988; Moss, 2002;Yazar and Omurtag, 2008).Currently, several *Fusarium* spp. have emerged which now threaten the productivity and safety of small grain cereal crops throughout the world (CAST, 2003; Dohnal *et al*., 2008; Lowe *et al*., 2012).

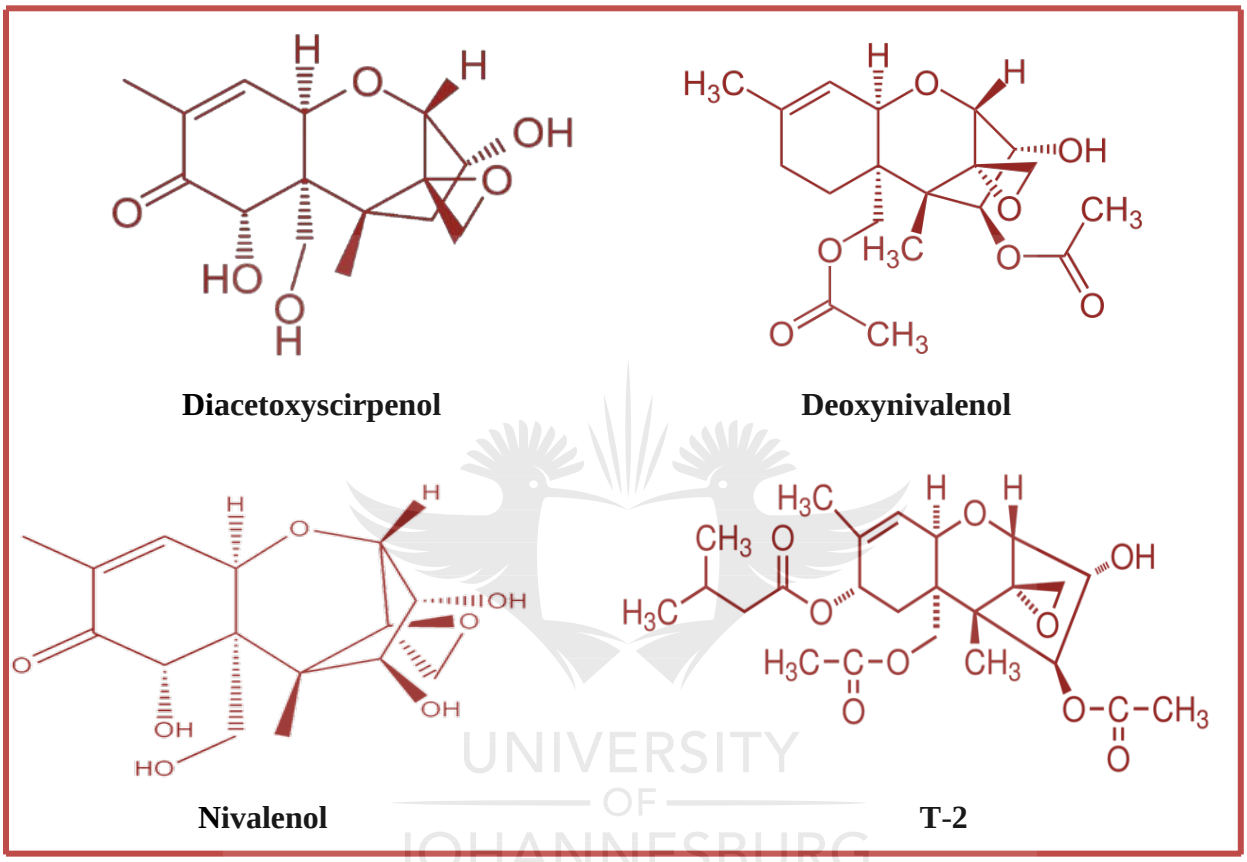


Figure 2.15 Chemical structure of Deoxynivalenol, Diacetoxyscirpenol, Nivalenol, (Lia *et al*., 2012) and T-2, (Thuvander *et al*., 1999).

To date, more than 180 naturally occurring trichothecenes have been isolated and characterized, but only a few have been found to contaminate food and feed (Eriksen, 2003; Larsen *et al*., 2004). These mycotoxins have been acknowledged as unavoidable contaminants of certain important agricultural commodities (Edwards *et al*., 2009; Fredlund *et al*., 2010; Mattila, 2010). Exposure to this family of mycotoxins may lead to multi-organ effects including vomiting and diarrhoea, weight loss, nervous disorders, cardiovascular alterations, immune-depression, homeostatic derangements, skin toxicity, decreased reproductive capacity, and bone marrow damage (Ueno, 1989; Franz *et al*., 1997; Wannemacher *et al*., 1991). The THs (DON, NIV, DAS

and T-2) are the best studied due to their potential misuse as biological weapon (Rosen and Rosen, 1982; Mirocha *et al*., 1983; Ueno, 1989).

Additionally, the first recognized THs mycotoxicosis reported was alimentary toxic aleukia (ATA) in Russia during World War II, an epidemic which affected a large number of people (CAST, 2003; SCF, 2001). Consequently, the incidence was linked to cereal grains which remained for extended periods on the field after normal harvest during warfare, often at oscillating temperatures, which led to the growth of *Fusarium* and production of THs. In this event, grain samples cultured revealed the presence of *F. sporotrichioides* isolates and were found to produce T-2 and its derivatives (Mateo *et al*., 2002). In generally, HT-2 toxins co-occur with T-2 toxins in infected grain products. Furthermore, HT-2 is a major metabolite of T-2 and T-2 is only differentiated from HT-2 by an acetyl group in the C-4 position. On the other hand, it is difficult to differentiate the effects of T-2 and HT-2 *in vivo* since HT-2 is rapidly formed after exposing an animal to T-2 (Eriksen and Alexander, 1998; Jestoi *et al*., 2008; Medina and Magan, 2011). T-2 and HT-2 are contaminants found in various cereal crops such as wheat, corn, barley, oats and rye and processed grains (malt, beer and bread) worldwide (SCF, 2001). T-2 and HT-2 have been reported to be produced by *F. sporotrichioides*, *F. poae*, *F. equiseti* and *F. acuminatum* (Creppy, 2002; Raju and Devegowda, 2000; D'Mello, 2003). Moreover, the severity of the mycotoxicosis was related to the duration of consumption of toxic grain. Wang *et al*. (1993) also reported the first human mycotoxicosis in China due to mouldy rice contaminated with *Fusarium* and T-2 toxin and a single ingestion of bread containing toxic flour in India (Bhat *et al*., 1989). According to Gyongyossy-Issa *et al*., 1985; Platt *et al*., 1989 and WHO, 1990, T-2 is well known to inhibit DNA, RNA and protein synthesis, mitochondrial function as well as other subcellular processes, cause death of eukaryotic cells and has a direct lytic effect on erythrocytes.

Deoxynivalenol is one of the most frequently found mycotoxins that commonly contaminates grain-based foods worldwide (Pestka, 2007). Similar to T-2 and HT-2, DON naturally occurs in various crops such as wheat, barley, oats, rye, rice and maize and is produced mainly by two important cereal pathogens: *F. graminearum* and *F. culmorum* (Pitt and Hocking, 1997b; Eriksen, 2003; Instanes and Hetland, 2004). Concurrent fungal contaminations with DON

production in the field is mainly dependent on weather conditions and is favoured by low temperatures and high humidity (Pestka and Smolinski, 2005). When ingested in high doses by agricultural animals, it causes nausea, vomiting, diarrhoea and can cause death; at lower dietary doses, pigs and other farm animals exhibit weight loss and anorexia (Rotter *et al*., 1996; Wijnands and van Leusden, 2000). Because DON is less toxic than other trichothecenes, e.g., T-2 toxin, higher doses are necessary for toxic effects (>3-5ppm)to manifest themselves but these are unlikely to be encountered in food (Pestka, 2007). Experimental animal studies have demonstrated effects on the immune system (Pestka *et al*., 2004), neuroendocrine effects of DON (Rotter *et al*., 1996). Pestka and Smolinski, (2005) also reviewed some detailed toxicity effects of DON on human and animal. Deoxynivalenol is sometimes called vomitoxin because of its strong emetic effects and its action as a feed refusal factor and it was first characterized and named following its isolation from *Fusarium* infected barley in Japan (Beardall and Miller, 1994; Miller *et al*., 2001).

Nivalenol (3, 4, 7, 15-tetrahydroxy-12, 13-epoxytrichothec-9-en-8-one) generally occurs together with FUS X and is one of the well-known mycotoxins among naturally occurring THs (Eriksen, 2003). *Fusarium cerealis* and *F. poae* are the main producers of NIV, but isolates of *F. culmorum* and *F. graminearum* are also able to produce nivalenol (Glenn, 2007). *Fusarium poae* is more widespread in Europe and has been reported an important producer of NIV in Sweden (Larsen *et al*., 2004). Nivalenol occurs in various cereal crops such as wheat, corn, barley, oats, and rye. In contrast to DON, NIV occurs more frequently in years with dry and warm growing seasons (SCF, 2000b). It has also been frequently detected in cereal grains and foods produced in Korea, China and other countries, and are thought to induce several food-borne diseases (Ito *et al*., 1986). Nivalenol is a potent inhibitor of protein, RNA, DNA synthesis in mammalian cells and causes necrosis of the proliferating cells *in vivo* (Ito *et al*., 1986). Because of this, NIV is especially toxic to rapidly dividing cells (Maragos *et al*., 2006) such as cells of lymphoid organs and intestinal mucosa (Tep *et al*., 2007). Nivalenol induces apoptosis in HL60 cells (Ueno *et al*., 1995).

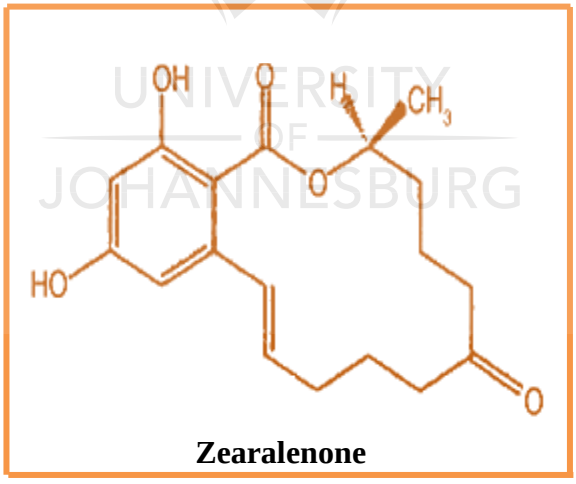
Diacetoxyscirpenol is naturally occurring in agricultural products (Mirocha *et al*., 1976) and is one of the most researched metabolite of the THs, it is produced by certain *Fusarium* species

such as *F. poae*, *F. semitectum*, *F. verticillioides*, *F. sporotrichioides*, *F. acuminatum*, *F. culmorum*, *F. crookwellense*, *F. venenotum*, *F. sambucinum*, *F. equiseti*, *F. graminearum*, *F. avenaceum*, *F. langsethiae* (Bauer *et al*., 1985; Omurtag *et al*., 2007). However, DAS was first isolated from cultures of *F. scirpi*, *F. equiseti* and *Gibberella intricans* (Swanson *et al*., 1982). Diacetoxyscirpenol is abundant in various cereal crops such as corn, barley, mixed feed samples and other grains from various regions in the world. In addition, co-occurrence of DAS and T-2 in animal feeds and human foods represent a health threat to humans and animals in some parts of the world (Wang *et al*., 1996). This metabolite possesses a wide-range of biological activity, including toxicity to fungi, plants, animals, and various mammalian tissue cultures (Bauer *et al*., 1985). Both in humans and animals the toxic effects of DAS appear similar including nausea, vomiting, diarrhoea, hypotension, neurological symptoms, chills and fever. Also, the haematopoietic system appears extremely sensitive, showing severe myelo-suppression. These symptoms in animals are independent of the route of dosing (Wang *et al*., 1996). Studies carried out by Thuvander *et al*. (1999) pointed out the role of DAS effectively they inhibit proliferation and immunoglobulin production in mitogen-stimulated human lymphocytes in a dose-dependent manner with limited variation in sensitivity between individuals. Diacetoxyscirpenol was shown in oesophageal hyperplasia but not in cancer in the rat (Craddock *et al*., 1987), and has also undergone clinical trials as a chemotherapeutic agent in cancer patients (Pronk *et al*., 2002).

2.5.8 Zearalenone

Zearalenone is described chemically as a phenolic resorcyclic acid lactone (Diekman and Green, 1992), mycotoxin that can be produced by several fungi of the genus *Fusarium*, in particular *F. graminearum*, *F. culmorum*, *F. cerealis*, *F. equiseti*, *F. semitectum*, *F. crookwellense*, which frequently colonize maize worldwide, but it may occur in oats, barley, wheat and sorghum (Wood, 1992; Gajecki *et al*., 2010). Zearalenone production is favoured by high humidity and low temperatures conditions and may also contaminate maize during storage if it is harvested with too much moisture and is not properly dried prior to storage (CAST, 2003). Zearalenone is a heat stable metabolite both during storage and processing, and it does not degrade at high temperatures (SCF, 2000c). It may co-occur with DON in grains such as wheat, barley, oats and corn and FBs in corn (Gonzalez *et al*., 1999). Research studies have shown that ZEA could co-occur with DON. Generally, DON is found in higher doses than ZEA when this occurs

(Osweiler, 1986). The natural occurrence of ZEA in a variety of agricultural commodities has been extensively reviewed by IARC, (1993a). The main cause of concern is that ZEA, previously known as F-2 toxin, is a non-steroidal oestrogenic metabolite that causes vulvovaginitis and oestrogenic responses in farm animals, especially in swine and possibly in humans (Wood, 1992; Placinta *et al*., 1999; Moss, 2002; Pillay *et al*., 2002). Livestock fed mouldy feeds containing ZEA may produce milk and milk products that contain these oestrogenic substances (Schoental, 1977). Oestrogenic agents can increase the plasma levels of cholesterol and triglycerides in females and an association between oral oestrogen use and myocardial infection and stroke has been described (Wallace *et al*., 1977). Its oestrogenic properties make exposure a concern for human health. The compound was shown in the 1980s to be produced on maize in the temperate regions of America, Europe and Asia. Also findings from South America, Africa and China have been reported, in these cases in different food and feed products (Krska *et al*., 2003). However, according to preliminary studies ZEA concentrations in different commercial food commodities vary significantly, and some products contain very high levels, depending on the climatic conditions (Zwierzchwski *et al*., 2004).The chemical structure of ZEA is shown below in Fig. 2.16.



Zearalenone

Figure 2.16 Chemical structure of Zearalenone, (Ouanesa *et al*., 2003).

Zearalenone has also been shown to be an immunotoxin, mutagenic, haemotoxic and hepatotoxic, however the mechanisms of toxicity are not fully understood (Zinedine *et al*., 2007). *In vivo* studies have revealed that ZEA is rapidly metabolized in animals and humans and eliminated mainly as water-soluble glucuronosides. Free and conjugated forms of ZEA have

been found in the milk of lactating cows under experimental conditions. That high oral doses of the toxin are required to elicit such a response indicating that consumption of contaminated feed by dairy cows would not result in a health hazard to humans (Wood, 1992). Zearalenone at amounts greater than normally encountered in field exposures (200 mg/kg of feed) does not adversely affect the reproductive potential of mature boars (Osweiler, 1986; Ruhr *et al*., 1983). ZEA and some of its metabolites have been shown to competitively bind to oestrogen receptors in a number of *in vitro* systems. Bindings to specific receptors have been displayed in uterus, mammary gland, liver and hypothalamus in different species (SCF, 2000c). Contamination of maize with ZEA is a threat to animal and public health and seriously reduces the quality of corn products. Fertility problems have been observed in animals such as swine and sheep (Krska *et al*., 2003).The most important toxicity effects of ZEA primarily include the urogenital system. Swine are the most commonly affected animals. Also, cattle, poultry and experimental rodents were affected (CAST, 2003). ZEA causes changes in the reproductive system of experimental animals such as mice, rats, guinea-pigs, hamsters, rabbits and domestic animals (SCF, 2000c).

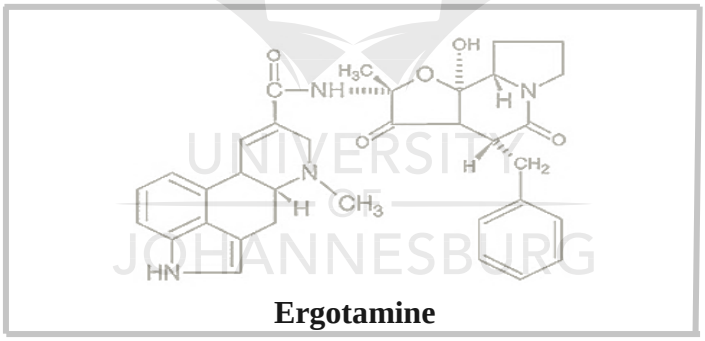
In humans, ZEA may be an important aetiologic agent of intoxication in young children or foetuses exposed to this metabolite, which results in premature puberties, and breast enlargement (CAST, 2003). In Puerto Rico, ZEA was found in the blood of children with precocious sexual development exposed to ZEA contaminated food (Bhat *et al*., 1997). Zearalenone was also found together with other *Fusarium* mycotoxins in "scabby grain toxicosis" in China (Luo, 1988). Due to economic losses engendered by ZEA and its impact on human and animal health, several strategies for detoxifying contaminated crops are crucial for food safety and have been described including physical, chemical and biological process (Zinedine *et al*., 2007; Kriszt *et al*., 2012).

2.6 OTHER MYCOTOXINS

Mycotoxins cause a diverse range of toxic effects because their chemical structures are very different from each other. The importance of these mycotoxins varies widely and is governed as much by the biology and ecology of the species concerned. In this event, other mycotoxins of great significance different from those described above are ergot alkaloids (EAs), moniliformin (MON) and penicillic acid (PCA) will be discussed herein.

2.6.1 Ergot alkaloids

The group of compounds termed ergot alkaloids (Fig. 2.17) are produced by a number of fungal genera such as *Acremonium*, *Balarisia*, *Aspergillus* and *Claviceps* (Panaccione *et al*., 2006; Schardl *et al*., 2006). The word ergot is derived from the old French word "argot" meaning cock's spur. However, the use of this term is associated to a disease caused by fungi belonging to the genus *Claviceps* on plants belonging to the grass family (EFSA, 2012b).This fungus is known to be more of a problem on some economically important cereals including rye, wheat, barley, oats, millet, sorghum and maize. Athough, rye is the most susceptible and produces alkaloids that can cause ergotism in humans and animals (Jayalakshmi and Jyothi, 2012). Ergotism is referred to the severe pathological syndromes affecting humans and animals that have ingested plant material containing ergot alkaloid, such as ergot-contaminated grains (Heritage *et al*., 1999). In the middle ages, the consumption of EA contaminated grains, bread caused severe epidemics of the condition ergotism, when was as St. Anthony's fire (EFSA, 2012b).



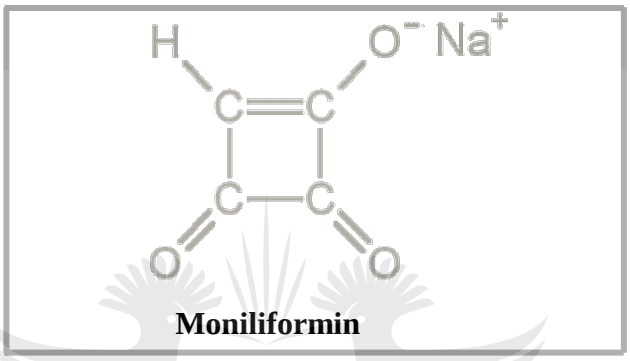
Ergotamine

Figure 2.17 Chemical structure of ergotamine, (Moss, 1996).

2.6.2 Moniliformin

The mycotoxin moniliformin was discovered by Cole *et al*. (1973) while screening for toxic products of a North American isolate of *F. verticillioides* cultured on maize and occurs as a sodium or potassium salt of (1-hydroxycyclobut-1-ene-3,4-dione, Fig. 2.18). Moniliformin is mainly produced by *F. proliferatum* and also by a number of other *Fusarium* genera that include *F. avenaceum* and *F. subglutinans* (Logrieco *et al*., 1995; Golinski *et al*., 1996). Furthermore, under different climatic conditions *F. proliferatum* and other *Fusarium* genera may have

different potential to produce MON in the field (Golinski *et al*., 2009). Moniliformin causes cardio-toxic effects in experimental and domestic animals, such as rats, chickens, turkey and ducks (D'Mello *et al*., 1999). In humans, MON has casually been associated with keshan disease, a myocardiac human impairment occurring in rural areas of China and South Africa (Transkei), regions with high maize and in a variety of maize-based and human foods and feeds worldwide (Battalico and Perrone, 1998; Valdes and Bullerman, 2000).

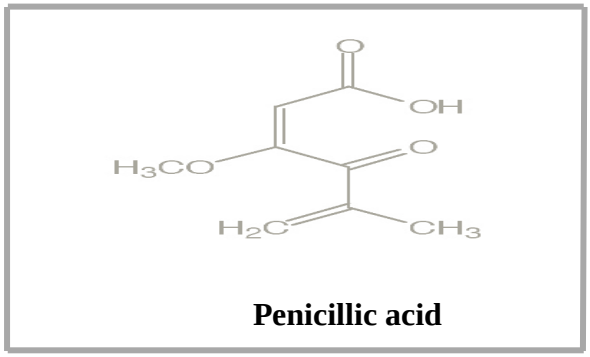


Moniliformin

Figure 2.18 Chemical structure of moniliformin, (Valdes and Bullerman, 2000).

2.6.3 Penicillic acid

Penicillic acid (Fig. 2.19) is a polyketide mycotoxin produced by several genera of *Aspergillus* and *Penicillium* (Sorenson and Simpson, 1986). Many feedstuff raw materials, such as sorghum, barley, oats, dried beans, wheat, maize, commercial tobacco and rice are able to produce PCA (Bhatnagar *et al*., 2002; Lei *et al*., 2010). It is one of the main mycotoxins in mouldy feedstuff and has toxic effect on livestock and poultry and probably humans due to food chain transmission (Pandiyan *et al*., 1990; Bernhoft *et al*., 2004; Keblys *et al*., 2004). This mycotoxin is toxic in experimental animals and has also been reported to be carcinogenic (Mori *et al*., 1984). According to Stoev *et al*. (2001) the interaction of penicillic acid and other mycotoxins, such as CTN, CPA, OTA and PAT on animals contributes to cumulative toxicity and enhance its toxicity.



Penicillic acid

Figure 2.19 Chemical structure of Penicillic acid, (Keblys *et al*., 2004).

2.7 MYCOTOXIN CONTROL AND PREVENTION

The hazards of mycotoxins in particular to humans and animals are well-recognized. In the recent years, there has been a great concern about the control and prevention of mycotoxin contamination particularly in maize, since maize is the staple food and agricultural commodity through the world. In addition, fungal species producing mycotoxin are extremely common, and they can grow on a wide range of substrates under a wide range of environmental conditions. For agricultural commodities, the severity of crop contamination tends to vary from year to year based on weather and other environmental factors. Moisture and temperature are the two main factors that have a crucial effect on fungal manifestation and toxin production. In the pre-harvest period, crops that have experienced significant stress whether it is from drought or maybe insect damage can succumb to fungal contamination. Aflatoxin, for example, is generally worst during drought years; the plants are weakened and become more vulnerable to insect damage (Dowd, 1998). Prior to harvest, preventive measures must begin with good agronomic practices including cultivating to improve plant vigour, irrigation to avoid moisture stress, harvesting at maturity and breeding programmes to improve genetic resistance to fungal invasion (CAST, 1989).

During post-harvest period, control of moisture and temperature of the stored commodity will mostly determine the degree of fungal activity (Christensen, 1974). Moisture content depends mostly on water content at harvest and can be amended by drying (efficient drying to <14% moisture content), aerating, and turning of the grain before or during storage. But then again, contamination associated with post-harvest stages of cleaning, drying, storage and processing need to be identified in terms of the type and amount of fungi and mycotoxins present in each

stage and in the by-products used for animal feeding (CAST, 1989; Blandino *et al*., 2004). Apart from methods that modify the fungal environment, there are other various ways available to inhibit fungal invasion either by chemical, biological and physical treatments (CAST, 2003; Cole, 1989).

Chemically, they can be controlled through the use of fungicides, mould inhibitors and pesticides for the control of fungi, as well as insects and pests in the field and during storage (Sauer and Burroghs, 1974; Dixon and Hamilton, 1981; Ryu and Holt, 1993). Agricultural commodities have also been treated with acids, alkalis, aldehydes, oxidizing agents and gases like chlorine, sulphur dioxide, ozone and ammonia (Goldblatt and Dollear, 1979) to control fungal infestation in grains.

Biological measures can be the use of high fungal resistant and insect varieties of crops (Bhatnagar *et al*., 1991) and the use of cats to prey on rodents in storage facilities. The Agricultural Research Science of the United States Department of Agriculture (USDA), (2005) have made several efforts to provide advanced technology necessary to reduce and eliminate hazards of naturally occurring toxins that are harmful to animals and humans. This was accomplished through biological control strategies using harmless bacteria to prevent the formation of mycotoxin in maize by *F. verticillioides*. The use of toxin binders such as hydrated sodium calcium, alumino silicate or a mannan-oligosaccharide (biological method) included in animal diets (Kubena *et al*., 1990; Savage *et al*., 1996). These substances have been found to bind toxins together in the alimentary canal and are eliminated them via faeces (Devegodwa *et al*., 1998; Carter, 2001). Another aspect through which the immuno-suppressive negative effects of mycotoxins can be minimized is through the inclusion of vitamin C and lysine in diets in order to stimulate the immune responses (Tudor and Petruta, 2001).

Physically, mycotoxin production is controlled through storage at low temperature, controlling relative humidity (Van Schothorst and Brooymans, 1982). Cleaning through sorting of mouldy seeds also falls within this category. Physical control measures are also important aspect through which the concentrations of mycotoxins can be reduced. As maize and maize-based products are consumed daily in the rural areas, there is likely to be high FB intake. However, such levels may

be reduced considerably during processing of maize flour into porridge. A significant reduction of FB was observed in maize in Benin by Fandoham *et al*. (2005) when grain was processed into food products by sorting, washing and fermenting. This may be through mixing of mycotoxin-contaminated feeds with freshly produced ones, although this may not be recommended in certain circumstances and also in some countries with stringent mycotoxin regulation laws. Other examples of physical measures are the use of monoclonal antibodies, which have been shown to have a high degree of specificity for T-2 toxin and was reported to be effective in neutralizing the cytotoxicity of this mycotoxin (Bhatnagar *et al*., 2002).

In some cases, a combination of these methods have been applied to control fungi, production of mycotoxins in foods and feeds, as well as the toxic effects they cause to livestock and humans. Although there are a number of ways through which the growth of fungi can be inhibited, it is not unlikely that some species may still persist, which in effect, may be toxigenic and thus produce toxins. Once mycotoxin contamination has occurred, it can be alleviated by a variety of predominant post-harvest measures including drying, physical separation and storing systems (FAO, 1997; Lopez-Garcia *et al*., 1999; Odamtten, 2001). A series of acidic, alkaline and neutral food additives were examined and found to be of potential use in the degradation of aflatoxins in corn and butter bean (Tabata *et al*., 1994).

2.8 CONCLUSION

It is known for many years that several food items derived from plants which are invaded by fungi in the field, during harvest and storage of the food item can concurrently contain different mycotoxins. On the other hand, fungi may also invade the food without the ability to produce mycotoxins. Therefore, identification of fungal contamination does not necessary confirm the presence of mycotoxins. What is more, even when mycotoxins are detected, it is not easy to associate them with acute and chronic illness. However, several studies have adequately demonstrated that mycotoxins are hazardous to humans and animals. In this case, maize and maize-based products play a significant role in our food chain and economy. Mycotoxins usually enter the body via ingestion of contaminated foods, but inhalation of toxigenic spores and direct dermal contact are also important routes. Subsequently, foodstuffs must be essentially controlled during food handling/processing and all mycotoxin analyses for the entire food chain has

importance for human health. It is crucial to continue to maintain food quality through minimizing the occurrence of these mycotoxins in maize and maize-based products. Research innovations, good agricultural practices and mycotoxin regulations will remain significant and reliable in food safety.

CHAPTER THREE

Fungal dissemination by housefly (*Musca domestica* L.) into various food commodities in the Gauteng Province, South Africa

ABSTRACT

Several insects that act as vectors including houseflies (*Musca domestica* L.) are often considered as the primary cause of fungal contamination in human foods and feeds. Houseflies are also involved in the transmission of bacterial pathogens that also poses a serious hazard to human health. Thus, the rural population of Gauteng Province of South Africa is at high risk from fungal exposure disseminated by houseflies. The study was therefore aimed to assess the role of houseflies in enhancing fungal contamination in various food commodities. Fungal isolates were primarily identified based on morphological characteristics by conventional identification methods and representative isolates confirmed by DNA sequencing. In a mycological study, a total of 729 fungal isolates of 15 genera including *Aspergillus*, *Fusarium*, *Penicillium*, *Alternaria*, *Chrysosporium*, *Cladosporium*, *Curvularia*, *Epicoccum*, *Eupenicillium*, *Moniliella*, *Mucor*, *Nigrospora*, *Rhizopus*, *Scopulariopsis* and *Yeasts* in decreasing order of prevalence were identified from the external surfaces of both female (84) and male houseflies (38), maize (15), porridge (19) and water (27) samples. The incidence rates of fungal contamination per total fungal count isolated were recorded with fungal load of 2 x 10^8 CFU/ml for houseflies, 2x 10^7 CFU/g for porridge, 1x 10^7 CFU/g for maize and 1 x 10^2 CFU/g for water. In addition, dominant fungal isolates of the female housefly samples were *A. flavus*, *F. verticillioides*, *P. verrucosum* and *M. suaveolens*, while *A. flavus*, *A. parasiticus*, *F. verticillioides*, *F. proliferatum* and *P. aurantiogriseum* were prevalent in male housefly samples. *Fusarium verticillioides*, *A. flavus*, *A. niger* and *P. oslonii* were the most prevalent species contaminating maize, porridge and water. The predominance of fungal contamination poses serious health concerns to the general community of Gauteng province. Looking into the adverse impact of fungi disseminated by houseflies, it is important to study the occurrence of fungi in maize, porridge and drinking water because they are capable of producing mycotoxins which are secondary metabolites of these filamentous fungi.

Key words: Housefly, food commodities, water, fungal isolation.

3.0 INTRODUCTION

Different types of insects invade most houses, including those that are accidental intruders that buzz against the window panes trying to escape into the open air. However, there are those that make human habitation their home, like the houseflies. Houseflies (*Musca domestica* Linnaeus. *Musca domestica* L.) are flying insects that are frequently found throughout the world (Fotedar, 2001; Graczyk *et al*., 2001; Kabkaew *et al*., 2007). They are not only irritating but also play a significant role as a route of pathogens to exposed food that may be transported to humans as well as to domestic animals, therefore they are regarded as a major pest problem to humans globally (Siri *et al*., 2005; Tilak *et al*., 2010). Large populations of *Musca domestica* L. may drastically cause food spoilage and contribute to substantial public health problems (Axtell, 1970; Axtell and Arends, 1990; Howard and Wall, 1996). Furthermore, pathogens and dirt are transmitted by houseflies from garbage and pit toilets (faeces) to human food. Contamination may occur both mechanically and through contaminated external body parts of the fly after consumption. Pathogens are transmitted through vomiting and defecation of the flies while on food (Zarrin *et al*., 2007; Vasan *et al*., 2008). Houseflies contaminate water and food, which can lead to them being ingested by humans. In this chain of microbial contamination, human faeces are important if not disposed of safely (Winblad, 1996). They are known to contain pathogens for a vast number of diseases such as cholera, dysentery, haematic carbuncle, poliomyelitis (Howard, 2001; Barin *et al*., 2010) and Shigellosis (caused by *Shigella* species) (Conner, 1966).

In addition to bacteria and virus, Costa and Oliver (1998) showed that there is also an association between houseflies and filamentous fungi. The spread of filamentous fungi by houseflies on to suitable substrates may result in production of secondary metabolites known as mycotoxins, which can cause harmful effects in humans. The mycotoxin producing fungi are of the genera *Aspergillus*, *Fusarium*, *Penicillium* species (Pitt and Hockings, 1997a) all of which produce spores that may be picked up by flies. The most significant mycotoxins produced by these fungi include: aflatoxins, ochratoxins, fumonisins, trichothecenes, T-2 toxin, deoxynivalenol, citrinin, patulin and zearalenone (Devegowda and Castaldo, 2000; Pitt and Hockings, 2009). These may be produced in food and food commodities where conditions are favourable for fungal growth. Consumption of such contaminated food can lead to acute and chronic toxicity, mutagenicity and teratogenicity, observed in human and animal health (Pitt, 2000b). Davari *et al*. (2012) verified

the association of fungi and houseflies collected from a slaughter house and a hospital in Iran using a different method and culture medium. However, research interest in the interaction of filamentous fungi and houseflies in South Africa is scanty, hence this study focuses on the isolation and identification of fungi isolated from *Musca domestica* L. in an attempt to elucidate their role in the dissemination of fungi in an African rural area. It is anticipated that this information will assist in improving the general health of the rural population.

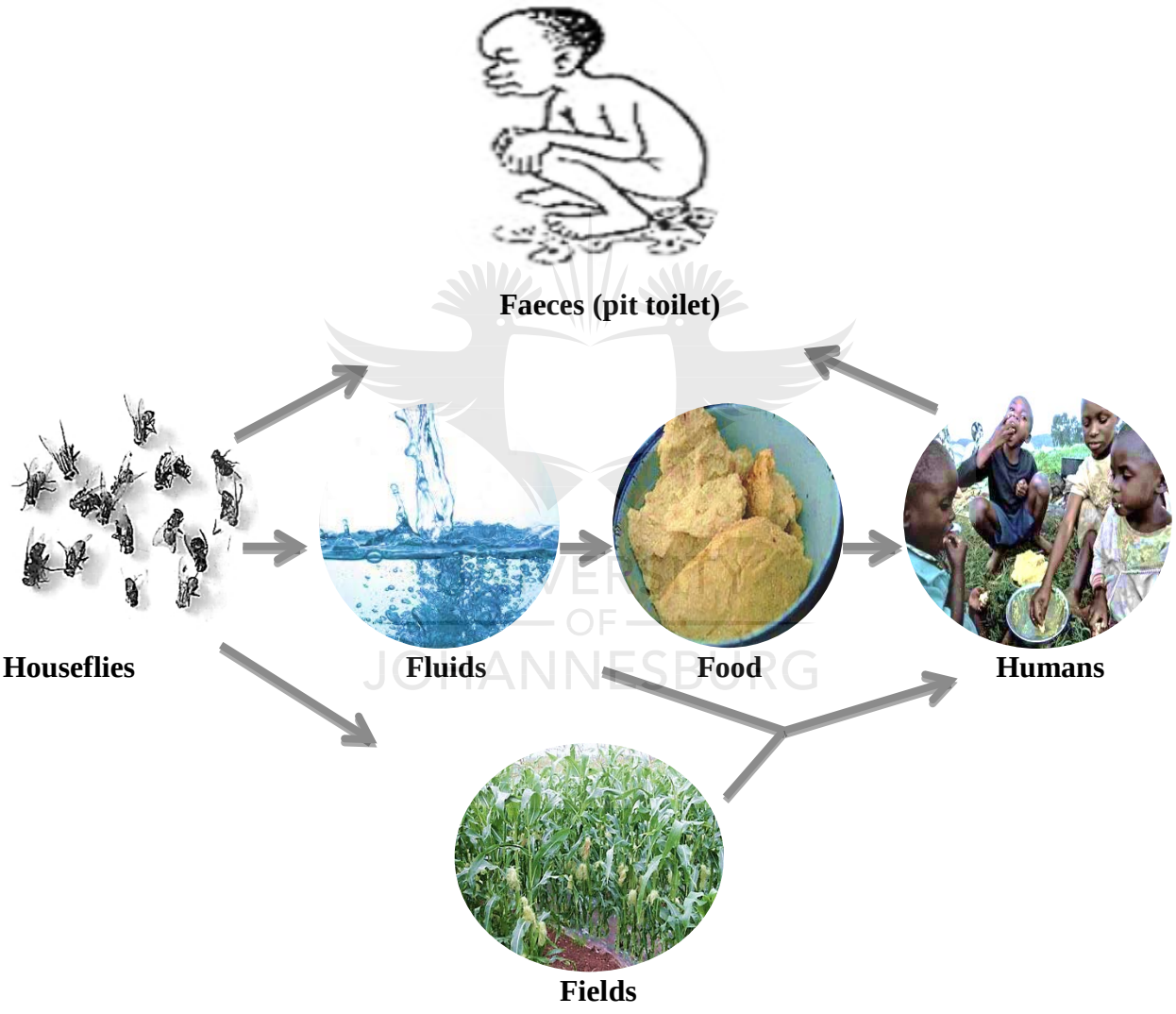


Figure 3.1 Typical entry route of fungal dissemination by houseflies (modified from Winblad, 1996).

3.1 RESEARCH MATERIALS AND METHODS

3.1.1 Materials

(a) ***Fly trap:*** Fly traps with a propylene material (19 cm in diameter and 25 cm height) were purchased from Marco Plastics, SA.

(b) ***Ringer's tablets and antibiotics*****:** Ringer's tables were purchased from (Merck, Germany), Streptomycin and Chloramphenicol (Sigma- Aldrich).

(c) ***Fungal screening culture media and identification*****:** Potato Dextrose Agar (PDA), Ohio Agricultural Experimental Station Agar (OAESA), Malt Extract Agar (MEA), Lactophenol blue solution, (Merck, Germany) and light microscope BX51 model, Ultra 20 soft imaging system (Olympus, Japan).

3.1.2 Installation of fly traps

To capture houseflies, into a separate lid a 51 mm diameter circle was drilled, to allow the flies to enter into a polystyrene cup. The lid was then stuck onto the underside of the fly trap's lid using a glue gun. A 4 mm drill bit was used to drill a star formation on the base of the polystyrene cup. The cup was clipped onto the lid that was stuck on the underside of the fly trap, so that it would contain the flies and ensured that they did not fall into the receptacle cup of the fly trap. Thereafter, the polystyrene cup was then clipped onto the receptacle cup that contained the bait solution, prepared by pouring a powdered Ultra Biomass Bait in the receptacle cup and adding 1 litre (L) of water. The assembled fly catcher appeals strongly to houseflies' natural instincts and provides a warm dwelling, while the bait solution provides them with irresistible pleasant food and it is non-toxic to humans. The fly traps which were placed in different households and pit toilets among the rural population in the Gauteng Province of South Africa. They were collected after 8 weeks. A schematic diagram of a fly trap is presented in Fig. 3.2.

3.1.3 Sampling and sample preparation

Samples of houseflies (122), maize (15), porridge (19) and water (27) were collected from different households. After capture, in a laminar flow chamber the houseflies were separated in the laboratory according to their gender (female and male) and were stored in sterile test tubes, ready for the isolation of fungi. After capture, houseflies of the spp. *M. domestica* L. were separated in the laboratory according to their gender (females and males) were stored in sterile

test tubes containing 9 ml ringers solution and thoroughly vortexed for 2 min to create wash from each housefly for fungal isolation purposes. These test tubes were then placed in a laminar flow chamber where the rest of the isolation of fungi steps was carried out. The maize and porridge samples were put in sealed plastic bags. All water samples were collected in sterile 1000 ml screw-capped polyethylene bottles. Due to the distance between the sampling area and the laboratory the samples were placed in cooler boxes until the analysis were done. Prior to analyses, maize samples were milled using a mechanical blender, while for the porridge samples were freeze-dried and further crushed into powder using a pestle and mortar.

Figure 3.2 Typical example of a fly trap.

3.1.4 Preparation of culture media

The culture media used were prepared according to Atlas (2004). Ringer's solution was prepared by dissolving 2 ringer's tablets in 1 litre (L) distilled water. Antibiotic solution, 1% each of streptomycin and chloramphenicol 1 gram (g) each were dissolved in 100 millilitre (ml) of sterile

distilled water. The solution was sterilized by passing through a 0.22 micromolar (µm) filter. Potato dextrose agar is recommended for *Fusarium* spp. isolation because of its colour production (under the mycelia mat) was prepared by dissolving 39 g of PDA powder in 1 L bottle filled with distilled water. Ohio Agricultural Experimental Station Agar (OAESA), for the isolation of individual fungi colonies was prepared by dissolving 5 g glucose, 2 g yeast extract powder, 1 g sodium nitrite, 0.5 g magnesium sulphate, 1 g di-potassium hydrogen phosphate, 1 g oxbile, 1 g sodium propionate and 20 g nutrient agar in 900 ml distilled water. Malt Extract Agar (MEA), a media for the isolation of *Penicillium* and *Aspergillus* spp., was prepared by dissolving 50 g of MEA granules in 1 L of distilled water. To prepare CYA, di-potassium hydrogen phosphate (1 g), yeast extract agar (5g), sucrose (30 g), agar agar powder (15 g) and Czapek concentrate (10 ml) prepared by dissolved 30 g of NaNO3, 5g KCl, 5g MgSO4.7H2O, 0.1 g FeSO4.7H2O, 0.1g ZnSO4.7H2O and CuSO4.5H2O in 100 ml purified water and made up to was dissolved in 1 L of distilled water. All culture media were sterilized in an autoclave at 121^{o}C for 15 mins and further cooled in a water bath to 50^{o}C. After which 8 ml of the antibiotic solution was added to each 1 L autoclaved media and mixed by gently shaking to suppress bacterial growth. Twenty ml of prepared media was then poured into each 9 cm sterile Petri dishes, allowed to cool and solidify.

3.1.5 Isolation fungi

Fungal isolation based on traditional culture and morphological characteristics were performed. In a laminar flow chamber, the group (>10 houseflies per test tube) of either female or male houseflies from one trap were transferred into test tube containing 9 ml of sterile Ringer's solution and thoroughly vortexed for 2 min to create a wash from each housefly for fungal isolation purposes. A serial dilution technique was then employed where 1ml of the wash solution was retrieved and diluted in 9 ml sterile ringer's solution and vortexed for 2 min; the suspension was further diluted in sterile ringer's solution down to 10^{-6}, for a total of 6 dishes for each sample. For maize and porridge, 1 g from each sample was weighed into a test tube and diluted in 9ml of sterile Ringer's solution, vortexed and serially diluted further to 10^{-6}. An aliquot of 1 ml of the dilutions from each tube was inoculated onto solidified PDA and OAESA media plates containing chloramphenicol and streptomycin to inhibit bacterial growth and spread on the surface of media using a sterile bent glass spreader according to Srivoramas *et al*. (2012)

with modification. Fungal isolation for water samples was performed according to Hageskal *et al*. (2006) whereby a volume of 10 ml of the samples were filtered through sterile 0.45µm nylon filters with 33mm diameter (Millipore). An aliquot of 1 ml of the filtered water was inoculated onto PDA media plates. These inoculated plates were incubated at a temperature of 25^{o}C for up to 7 days and then checked for fungal colonies. The number of colonies on the agar plates was expressed as number of CFU per 1 ml for houseflies and water samples and per 1 g for maize and porridge samples. After colony count, single spore isolation from houseflies, maize, porridge and water were sub-cultured on PDA, Czapek yeast extract agar and malt extract agar to obtain a pure culture and incubated at 25^{o}C, reactivating their macro-morphological characteristics for identification of the various genera. Only PDA medium was used to subculture single spores from water samples.

3.1.6 Identification of fungal isolates through macroscopic and microscopic characters

For houseflies, each isolated colony was identified according to the following parameters: location of housefly capture, pool number of the macerated houseflies and number of the colony in each Petri dish. An optical microscope (Olympus CX40, Micro-Instruments New Zealand, Ltd) was used to observe the micro-morphological characteristics for species identification. The isolated pure fungal colonies were stained with lactophenol blue for mounting between the slides and cover slides. The macro- and microscopic characteristics of the genera *Fusarium* were identified using the keys of Nelson *et al.* (1983); Leslie and Summerell (2006) and Diba *et al*. (2007). While those of the genera *Aspergillus*, *Penicillium*, *Alternaria*, *Cladosporium* and other fungal genera were identified according to the description of Pitt and Hocking (1997c), Pitt *et al.* (2000b); Pitt (2000a) and Klich (2002a). In a case where the morphological characteristics of individual isolates and identified fungal isolates using conventional technique, isolates were sent to Inqaba Biotechnological Industries, (Pty) Ltd, Pretoria, South Africa for confirmation purposes via Polymerase Chain Reaction **(**PCR) analysis following the method described by Samson *et al*. (2004).

3.1.7 DNA Extraction, Polymerase Chain Reaction (PCR) and Sequencing

3.1.7.1 DNA extraction

Genomic DNA analysis was performed using a Fungal/Bacterial DNA extraction kit (Zymo Research Corporation, Southern California, USA). The freeze-dried cultures were allowed to

stand for 1 hr at room temperature followed by DNA extraction. In this case, about 60 mg of sample was mixed with 200 µl of phosphate buffer saline (PBS) contained in a 1.5 ml ZR Bashing Bead™ lysis tube. The lysis tube was placed in a beater and processed for 5 mins, followed by centrifugation at 10,000 *g* for 1 min. The supernatant was transferred to a Zymo-Spin™ IV spin filter in a 1.5 ml Eppendorf tube and again centrifuged at 7,000 *g* for 1 min. The content was filtered into a collection tube and 1,200 µl of fungal/bacterial DNA binding buffer added and vortexed. Extraction mixture (800 µl) was transferred to a Zymo-Spin™ IIC column in the collection tube which was again centrifuged at 10,000 *g* for 1 min with the supernatant discarded (x2). A 200 µl of DNA pre-wash buffer I aliquot was added to the Zymo-Spin™ IIC column in a new collection tube and centrifuged at 10,000 *g* for 1 min. The filtrate was discarded, while retaining the column, which was then placed into a new tube, 500 µl fungal/bacterial DNA wash buffer II was added to the Zymo-Spin™ IIC column and again centrifuged at 10,000 *g* for 1 min. The Zymo-Spin™ column was transferred to a sterile 1.5 ml Eppendorf tube and 100 µl DNA elution buffer was added directly to the column matrix. This was then centrifuged at 10,000 *g* for 30 seconds (secs) to elute the DNA.

3.1.7.2 Polymerase Chain Reaction analysis

Fusarium isolates were identified by isolating the translation elongation factor (TEF) 1α region following the sequence obtained from different databases. The primer sequences used were described by O'Donnell and Cigelnik, (1997) designed in conserved 5' and 3' regions. The two primers: ITS-1; 5'-TCC GTA GGT GAA CCT GCG G-3' (forward) and ITS-4; 5'-TCC TCC GCT TAT TGA TAT GC -3' (reverse) resulted in the amplification of a 450 bp elongation factor product. The primers were synthesized at a 0.01 µM scale and purified using reverse-phase cartridge purification (Inqaba Biotechnical Industries (Pty) Ltd, South Africa). These primers were resuspended in 2 µM TE buffer prepared from a stock solution concentration of 100 µM.

The PCR was performed using the Fermentas 2 X PCR mix (Fermentas Life Science, Lithuania). The PCR mix for each sample consisted of 25 µl of 2 X PCR mix, 1 µl of each 2 µM primer, 1 µl of DNA (final concentration of 10 µM), and constituted to a final volume of 50 µl with nuclease free water. A negative control, containing all of the reagents except the DNA was prepared. The PCR was performed using an Eppendorff 96-well Thermocycler (Eppendorff, USA). The PCR

cycling conditions was set as follows: Pre-dwelling at 95^{o}C for 3 mins, 35 cycles denaturation at 95^{o}C for 1 min, annealation at 58^{o}C for 45 secs, extension at 72^{o}C for 1 min 30 secs, post-dwelling at 72^{o}C for 10 mins and held at 4^{o}C until samples were retrieved.

3.1.7.3 Agarose Gel DNA Electrophoresis

The preparation of 2% agarose gel was prepared by dissolving 2 g of agarose (Fermentas Life Science, Lithuania) in 98 ml 1 X TBE buffer (Fermentas Life Science, Lithuania) and then boiled. The solution was cooled to approximately 60°C. Ethidium bromide (3 µl) (Sigma-Aldrich, ST Louis, MO, USA) was added to the solution and thoroughly mixed. The agarose solution was poured into a casting chamber and the combs with 10 wells inserted. The chambers of the running system were filled with 1 X TBE buffer (Fermentas Life Science, Lithuania). Each PCR product (2 µl) was mixed with 10 µl of 6 X orange loading dye (Fermentas Life Science, Lithuania) and loaded into the wells. The chamber was closed and ran at 70 V for 15 mins. The PCR product was viewed using the Vacutec Gel documentation system and the product size was confirmed by comparing it to the Middle Range FastRuler (Fermentas Life Science, Lithuania).

3.1.7.4 Sequencing of the PCR Products

Polymerase Chain Reaction products obtained were cleaned using shrimp alkaline phosphatase and *E. coli* exonuclease I (Fermentas Life Sciences, Lithuania). The purity of the DNA was confirmed by running a 2% agarose gel (as described above). Automated DNA sequencing was performed using the SpectruMedix model SCE 2410 automated DNA sequencer (SpectruMedix, State College, PA).

Aspergillus and *Penicillium* isolates for DNA sequencing were diluted and PDA medium was used to subculture isolates for 7 days at 25^{o}C. The mycelia was scraped, transferred into a 0.5 ml sterile screw-cap vial containing 200 µl of ringer solution, freeze-dried and stored at -40^{o}C until analyzed. A FastDNA® Kit (Bio101, Carlsbad, USA) was used to isolate the genomic DNA according to the manufacturer's recommendations. Primers Bt2a and Bt2b (5´-GGTAACCAAATCGGTGCTGCTTTC), (5´-ACCCTCAGTGTAGTGACCCTTGGC) were used to amplify the β-tubulin genes, while the PCR reactions was performed in 50 µl reaction

mixture prepared by mixing 1 µl genomic DNA (10 ng/µl), 5 µl PCR buffer, 30 µl ultra-pure sterile water, 10 µl dNTP (1mM), 1 µl of each primer (50 pmol/µl) and 1µl Taq polymerase (2.5U/µl DNA) (SpaeroQ, Leiden, The Netherlands). A GeneAmp PCR System 9700 (AB, Applied Biosystems, Nieuwerkerk a/d Yssel, The Netherlands) was used to amplify the β-tubulin template, programmed for 5 cycles of 1 min denaturation at 94^{o}C, followed by primer annealing for 90 secs at 68^{o}C and extension for 2 mins at 72^{o}C and drop in annealing temperature of 1^{o}C/cycle, followed by 25 cycles of denaturation at 94^{o}C for 1 min, followed by primer annealing for 90 secs at 64^{o}C, extension for 2 mins at 72^{o}C and a final 10 mins elongation step at 72^{o}C. After amplification, a commercial GFX column, PCR DNA purification Kit (Amersham, Bioscience, Roosendaal, The Netherlands) was used to remove excess primers. The dNTP's and purified PCR fragments were re-suspended in 50 µl 1X TE buffer (10 mM Tris-HCl, 1 mM Na_2EDTA, pH 8.0) containing RNAse A at 20 µg/ml. Samples were incubated at 37^{o}C for 30 mins and then extracted with 300 µl of phenolz/CHCl3/isoamyl alcohol. The aqueous phase (~300 µl) was transferred to another tube and 25 µl of 7.5 M ammonium acetate and 125 µl of ethanol was added, mixed and incubated for 30 mins at -20^{o}C. The samples were then centrifuged at 4^{o}C for 15 mins at 12,000 x g, purified PCR fragments were then rinsed with 95% ethanol, air dried and re-suspended in 100 µl of 1X TE. Quality and quantity of the DNA obtained were determined by subjecting a portion of the preparation to agarose gel electrophoresis and UV spectrophotometry, whereby a fraction of the DNA preparations was subjected to gel electrophoresis.

The primers were used to sequence the PCR products in both directions with a DYEamic ET Terminator Cycle Sequencing Kit (Amersham, Bioscience, Roosendaal, and The Netherlands). The cycle sequencing reaction mixture (10 µl) used consisted of 1 µl template DNA (10 ng/µl), 4 µl Dye terminator RR mix, 4 µl ultra-pure sterile water and 1 µl primer (4 pmol/µl). The reactions was then run in a GeneAmp PCR System 9700 run in 9600 mode (AB, Applied Biosystems, Nieuwerkerk a/d Yssel, The Netherlands), previously programmed for 25 cycles of 10 secs denaturation at 96^{o}C, followed by primer annealing for 5 secs at 50^{o}C and extension for 4 mins at 60^{o}C. Purification of sequencing products was done as recommended by the manufacturer with Sephadex G-50 superfine columns (Amersham, Bioscience, Roosendaal, the Netherlands) in a multiscreen HV plate (Millipore, Amsterdam, the Netherlands) and with

MicroAmp Optical 96-well reaction plate (AB, Applied Biosystems, Nieuwerkerk a/d Yssel, The Netherlands). Samples were then analyzed on an ABI PRISM 3700 Genetic analyzer (AB, Applied Biosystems, Nieuwerkerk a/d Yssel, The Netherlands). The forward and reverse sequences were assembled using the programmes SeqMan and EditSeq from the LaserGene package (DNAStar Inc. Madison, WI). Alignments of the partial β-tubulin gene sequences data were calculated using a software package BioNumerics (Applied Maths BVBA, Saint Martens-Latem, Belgium) and adjustments made manually with the aid of an eye to maximize homology.

3.1.8 Statistical analysis

Mean concentrations of fungi on the external surfaces were calculated for the females and males houseflies by dividing the total number of CFU by the number of plates from each household (house and pit toilets). The plates were incubated at 25°C for 7 days, the colonies were counted, and results were expressed as CFU/ml and CFU/g.

3.2 RESULTS

Figure 3.1 represents seven days old colonies of some isolated spp. from analysed samples, grown on different culture media. Fungal contamination of houseflies, maize, porridge and water was analyzed based on the incidence rates of total fungi and fungal load per total fungal count data presented in Table 3.1. Mycological analysis in houseflies revealed that in total, 497 fungal isolates were identified from 122 captured housefly samples amongst which, 84 were female and 38 male. Results on houseflies revealed that 72 female and 38 male housefly samples were captured from different households and only 12 samples of female flies were captured from pit toilets. In addition to the mycological analysis in houseflies, a total of 232 isolates belonging to the same genera as those from houseflies were also isolated from maize (146), porridge (42) and water (44), results presented in Table 3.1.

Overall, mycological analysis of female and male houseflies from the external surfaces showed a wide range of fungi identified as *Aspergillus* species (spp.), *Fusarium* spp., *Penicillium* spp., *Alternaria* spp., *Chrysosporium* spp., *Cladosporium* spp., *Curvularia* spp., *Epicoccum* spp., *Eupenicillium* spp., *Moniliella* spp., *Mucor* spp., *Nigrospora* spp., *Rhizopus* spp., *Scopulariopsis* spp. and *Yeasts* spp. (Table 3.1). As shown in Table 3.1, *Fusarium* spp., *Penicillium* spp. and

Aspergillus spp. were the most predominant species in maize, while *Aspergillus* spp., *Penicillium* spp. and *Moniliella* spp. were frequent in porridge. On the other hand, *Aspergillus* spp. and *Penicillium* spp. were the only spp. isolated from water samples.

From the *Aspergillus* spp. captured from households, results revealed that 108 of those fungi were isolated from female houseflies, while 64 were isolated from male houseflies (Table 3.3). Eighty-five *Fusarium* species were isolated from the female and male houseflies captured in the households, (Table 3.3).

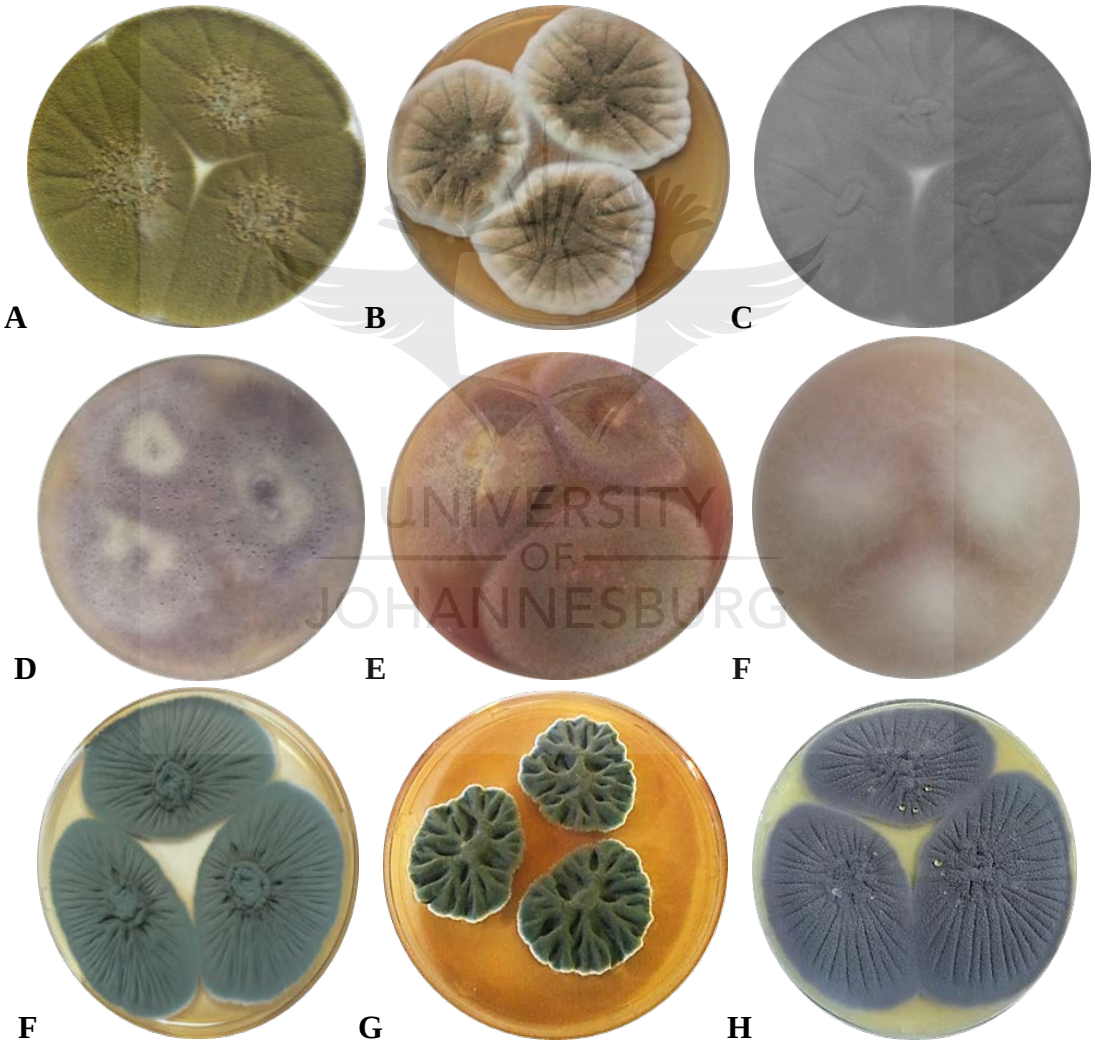


Figure 3.3 Macroscopical characters on different agar media (A-C): *A. flavus, A. wentii* and *A. fumigatus* mycelia growth on CYA; (D-F): *F. verticillioides, F. graminearum* and *F. proliferatum* growth on PDA; (G-I): *P. expansum, P. janthinellum* and *P. aurantiogriseum* growth on MEA isolated.

Table 3.1 Incidence rates of total fungi and fungal contamination per total fungal count isolated from houseflies, maize, porridge and water samples from Gauteng Province

	Houseflies			Maize			Porridge			Water		
Isolated species	**No. of isolates**	**%**	**CFU/g[a]**	**No. of isolates**	**%**	**CFU/g**	**No. of isolates**	**%**	**CFU/g**	**No. of isolates**	**%**	**CFU/g[b]**
Aspergillus spp.	186	37	4×10^6	19	13	4×10^5	18	43	2×10^6	37	84	6×10^1
Fusarium spp.	85	17	8×10^6	66	45	2×10^6	-	-				
Penicillium spp.	106	21	4×10^6	42	29	2×10^6	7	17	5×10^6	7	16	4×10^1
Alternaria spp.	7	1.4	3×10^5	-	-	-	-	-	-	-	-	-
Chrysosporium spp.	8	2	10×10^6	-	-	-	-	-	-	-	-	-
Cladosporium spp.	1	0.2	2×10^4	7	5	2×10^6	4	10	8×10^5	-	-	-
Curvularia spp.	2	0.4	6×10^4	-	-	-	-	-	-	-	-	-
Epicoccum spp.	6	1	3×10^6	-	-	-	-	-	-	-	-	-
Eupenicillium spp.	6	1	5×10^6	-	-	-	-	-	-	-	-	-
Moniliella spp.	43	9	5×10^6	4	3	1×10^6	7	17	2×10^6	-	-	-
Mucor spp.	11	2	5×10^7	2	1	2×10^6	5	12	4×10^6	-	-	-
Nigrospora spp.	7	1	5×10^6	-	-	-	-	-	-	-	-	-
Rhizopus spp.	8	2	2×10^6	3	2	2×10^6	-	-	-	-	-	-
Scopulariopsis spp.	8	2	5×10^7	3	2	9×10^5	-	-	-	-	-	-
Yeasts spp.	13	3	6×10^6	-	-	-	1	2	2×10^6	-	-	-
Total	**497**	**100**	**2×10^8**	**146**	**100**	**1×10^7**	**42**	**100**	**2×10^7**	**44**	**100**	**1×10^2**

Note: [a]CFU/ml: Colony forming unit per ml of sample; [b]CFU/g= Colony forming unit per gram of sample; No. = number.

From female houseflies, *Fusarium* spp. identified included *F. verticillioides, F. proliferatum, F. oxysporum, F. culmorum, F. semitectum, F. sporotrichioides, F. equiseti, F. graminearum, F. nivale, F. poae* and *F. avenaceum*. Furthermore, incidences of *Fusarium* species from male houseflies were *F. proliferatum, F. verticillioides, F. culmorum, F. oxysporum, F. avenaceum, F. poae* and *F. graminearum*. For *Fusarium* spp. isolated from houseflies captured from the toilets, *F. verticillioides* was the only fungi identified from female houseflies (Table 3.2). Results in Table 3.3 reveals that *Penicillium* spp. was also amongst the most prevalent fungi, which were isolated from houseflies captured from households. However, there were only four isolates identified from female houseflies captured from toilets; *P. aurantiogriseum, P. brevicompactum, P. crustosum* and *P. janthinellum*. In addition, to *Aspergillus, Fusarium* and *Penicillium* species isolated from female houseflies captured from households and toilets, *Moniliella* spp. (*Moniliella suaveolens*), *Yeasts* (*Candida krusei* and *paralopsis*), *Nigrospora* spp. (*Nigrospora oryzae*), *Chrysosporium* spp. (*Chrysosporium fornicola*), *Epicoccum* spp. (*Epicoccum nigrum*), *Eupenicillium* spp. (*Eupenicillium javinicium*), *Mucor* spp. (*Mucor plumbeus, racemosus, piriformis* and *circinellioides*), *Scopulariopsis* spp. (*Scopulariopsis brevicaulis*), *Rhizopus* spp. (*Rhizopus oligosporus* and *microspores*) and *Alternaria* spp. (*Alternaria infectoria*) were identified (Table 3.2).

Interestingly, similar isolates belonging to the same genera of *Aspergillus* isolated from houseflies namely, *A. carbonarius, A. flavus, A. niger, A. ochraceus, A. parasiticus* were also isolated in maize, porridge and water samples. However, *A. carbonarius* and *A. parasiticus* were the only fungi that were not isolated from porridge, Table 3.3. The genus *Fusarium* was also frequently isolated from maize, accounting for 66 of the 146 fungal isolates reported herein with *F. proliferatum* being the most dominant followed by *F. oxysporum* and *F. culmorum* as seen in Table 3.3. The *Penicillium* members accounted for 42 of the total 146 fungi isolated with *P. oslonii* being the most dominant in maize. In addition, *P. verrucosum* and *P. oslonii* were the most frequently isolated in 7 of the total 42 and 44 fungi isolated in porridge and water respectively. This study also revealed the presence of other genera of fungi such as *Cladosporium herbarum, Moniliella suaveolens, Mucor plumbeus, Rhizopus microspores* and *Scopulariopsis brevicaulis* in maize, while the isolates such as *Cladosporium herbarum, Candida krusei, Moniliella suaveolens* and *Mucor plumbeus* were only isolated from porridge.

Table 3.2 Incidence rates of fungal contamination with *Aspergillus*, *Fusarium*, *Penicillium* and other species in houseflies from Gauteng Province

	Households				Toilets			
	Female		Male		Female		Male	
Isolated species	**No. of samples**	**(%)**	**No. of samples**	**(%)**	**No. of samples**	**(%)**	**No. of samples**	**(%)**
***Aspergillus* species**								
A. candidus	1	1	-	-	-	-	-	-
A. carbonarius	8	4	8	4	1	1	-	-
A. clavatus	3	2	2	1	-	-	-	-
A. flavus	40	22	23	12	5	3	-	-
A. fumigatus	5	3	6	3	1	1	-	-
A. niger	19	10	6	3	4	2	-	-
A. ochraceus	11	6	4	2	2	1	-	-
A. oryzae	2	1	-	-	1	1	-	-
A. parasiticus	12	6	9	5	-	-	-	-
A. ustus	6	3	4	2	-	-	-	-
A. wentii	1	1	2	1	-	-	-	-
Total	**108**	**59**	**64**	**33**	**14**	**8**	-	-
***Fusarium* species**								
F. avenaceum	1	1	3	4	-	-	-	-
F. culmorum	4	5	5	6	-	-	-	-
F. equiseti	2	2	-	-	-	-	-	-
F. graminearum	2	2	1	1	-	-	-	-
F. nivale	2	2	-	-	-	-	-	-
F. oxysporum	8	9	4	5	-	-	-	-
F. poae	2	2	3	4	-	-	-	-
F. proliferatum	12	14	6	7	-	-	-	-
F. semitectum	3	4	-	-	-	-	-	-
F. sporotrichioides	3	4	-	-	-	-	-	-
F. verticillioides	17	20	6	7	1	1	-	-
Total	**56**	**65**	**28**	**34**	**1**	**1**	-	-
***Penicillium* species**								
P. aurantiogriseum	10	9	8	8	4	4	-	-
P. brevicompactum	11	10	5	5	1	1	-	-
P. citrinum	3	3	2	2	-	-	-	-
P. crustosum	9	8	2	2	1	1	-	-
P. expansum	1	1	-	-	-	-	-	-
P. janthinellum	10	9	4	4	1	1	-	-
P. oslonii	6	6	4	4	-	-	-	-
P. sclerotiorum	1	1	-	-	-	-	-	-
P. verrucosum	16	15	7	7	-	-	-	-
Total	**67**	**62**	**32**	**32**	**7**	**7**	-	-
Other species								
Alternaria infectoria	4	3	3	3	-	-	-	-
Chrysosporium fornicola	4	3	2	3	-	-	-	-
Chrysosporium inops	-	-	-	-	1	0.8	-	-
Cladosporium herbarum	-	-	1	0.8	-	-	-	-
Curvularia lunata	-	-	2	2	-	-	-	-
Epicoccum nigrum	5	4	-	-	1	0.8	-	-
Eupenicillium javinicium	4	3	-	-	2	2	-	-
Moniliella suaveolens	26	21.6	13	10.8	4	3	-	-
Mucor circinellioides	1	0.8	1	0.8	-	-	-	-
Mucor piriformis	1	0.8	-	-	-	-	-	-
Mucor plumbeus	2	2	4	3	-	-	-	-
Mucor racemosus	2	2	-	-	-	-	-	-
Nigrospora oryzae	7	6	-	-	-	-	-	-
Rhizopus microsporus	5	4	1	0.8	-	-	-	-
Rhizopus oligosporus	1	0.8	-	-	-	-	-	-
Rhizopus stolonifer	-	-	1	0.8	-	-	-	-
Scopulariopsis brevicaulis	5	4	2	2	1	0.8	-	-
Candida krusei	11	9	-	-	1	0.8	-	-
Candida paralopsis	1	0.8	1	0.8	-	-	-	-
Total	**79**	**64.8**	**31**	**27.8**	**10**	**8.2**	-	-

Note: Total number of *Aspergillus* species = 186; *Fusarium* species = 85; *Penicillium* species = 106 and other species = 120.

Table 3.3 Incidence rates of fungal contamination with *Aspergillus*, *Fusarium*, *Penicillium* and other species in maize, porridge and water from Gauteng Province

	Maize		Porridge		Water	
Isolated species	**Number of samples**	**Percentage (%)**	**Number of samples**	**Percentage (%)**	**Number of samples**	**Percentage (%)**
***Aspergillus* species**						
A. carbonarius	4	21	1	4	-	-
A. flavus	6	32	7	59	10	27
A. niger	3	16	4	15	18	49
A. ochraceus	1	5	1	4	-	-
A. parasiticus	5	26	5	19	9	24
Total	**19**	**100**	**18**	**100**	**37**	**100**
***Fusarium* species**						
F. avenaceum	4	6	-	-	-	-
F. culmorum	10	15	-	-	-	-
F. graminearum	7	10.5	-	-	-	-
F. nivale	6	9	-	-	-	-
F. oxysporum	11	17	-	-	-	-
F. proliferatum	7	10.5	-	-	-	-
F. semitectum	3	4.5	-	-	-	-
F. sporotrichioides	3	4.5	-	-	-	-
F. verticillioides	15	23	-	-	-	-
Total	**66**	**100**	-	-	-	-
***Penicillium* species**						
P. aurantiogriseum	5	12	1	14	-	-
P. brevicompactum	3	7	-		-	-
P. citrinum	7	17	1	14	-	-
P. crustosum	1	2	-	-	-	-
P. expansum	5	12	-	-	-	-
P. janthinellum	4	10	1	14	-	-
P. oslonii	12	28	2	29	5	71
P. verrucosum	5	12	2	29	2	29
Total	**42**	**100**	**7**	**100**	**7**	**100**
Other species						
Cladosporium herbarum	7	37	4	24	-	-
Candida krusei	-	-	1	6	-	-
Moniliella suaveolens	4	21	7	41	-	-
Mucor plumbeus	2	11	5	29	-	-
Rhizopus microsporus	3	15.5	-	-	-	-
Scopulariopsis brevicaulis	3	15.5	-	-	-	-
Total	**19**	**100**	**17**	**100**	-	-

Note: Total number of isolates in maize: *Aspergillus* species = 19; *Fusarium* species = 66; *Penicillium* species = 42; other species = 19. Total number of isolates in porridge: *Aspergillus* species = 18; *Penicillium* species = 7; other species = 17. Total number of isolates in water: *Aspergillus* species = 37; *Penicillium* species = 7.

To this end, mycological review in this study revealed the similarity in the taxonomic composition of fungi in houseflies and those of food commodities. The study also revealed that none of the samples analysed were free of fungal contamination. Additionally, incidence rates of co-contamination of *A. flavus*, *A. niger*, *A. parasiticus*, *F. verticillioides*, *F. oxysporum*, *F. proliferatum*, *F. culmorum*, *P. aurantiogriseum*, *P. verrucosum* and *P. oslonii* were observed particularly in houseflies and maize samples. *Aspergillus niger* and *A. flavus* were the

commonest of co-contamination observed in porridge and water. Raw data for fungal species and fungal contamination isolated on individual samples in this study is presented in Appendix I.

3.3 DISCUSSION

The main objective of this study was to isolate and identify the fungi on the external body surface of the houseflies, maize, porridge and water and to establish the correlation between the fungal load in houseflies and that of foodstuffs. In this study, approximately 729 fungal isolates were identified from houseflies (*Musca domestica* L.), maize, porridge and water, comprising 15 different fungal genera. It was observed that fungi belonging to the genera of *Aspergillus*, *Penicillium*, *Fusarium*, *Moniliella* and *Cladosporium* spp. were the most frequently isolated (Table 3.1). However, the most commonly occurring fungi in all sample types were identified as *A. flavus*, *P. verrucosum*, *P. oslonii*, *F. verticillioides*, *Moniliella suaveolens* and *Cladosporium herbarum*, results presented in Table 3.2 and Table 3.3. A concern in this study is that more houseflies were captured in households than in pit toilets. This may be attributed to the fact that, in the households there might be a wide selection of substrates favouring egg-laying (as a results of cooking). The household also provide a safe environment, away from predators. Another reason could be that houseflies are usually attracted to light and odour, and avoid darkness and dark surfaces (Keiding, 1986). The observation of different fungal genera isolated could be that the temperature in the households favours the growth of fungi since fungi can grow and be amplified indoors (Christensen and Sauer, 1982) although the history of houseflies in the study area is not known. Another explanation for the variety of fungal species might be that the houseflies were captured during February to mid-March where, temperatures (hot and humid) during that time in the study area (Gauteng Province) are suitable for larvae survival and fungal growth explaining the higher frequencies of the different genera found. Furthermore, in rural areas where subsistence farming is practiced the soil is the first environment for fungi and one of the important reservoirs for all types of fungi (Aghamirian and ghiasian, 2013). A large amount of stored maize was contaminated by houseflies, the key factors in the transmission of different types of fungi in stored maize (Sinha *et al*., 1998). Furthermore, the presence of houseflies in stored maize may lead to increased incidence of storage fungi particularly *Aspergillus* and

Fusarium. These mycoflora have been reported hidden in maize that can serve as nutrient sources for housefly development (Allotey, 2001).

Steinhaus, (1946) highlighted the importance of temperature and relative humidity conditions in the occurrence of fungal species. A moist environment favours germination and hastens the development of fungal spores. According to Hardin *et al*. (2003), a greater number of fungal species found in determined places could be attributed to climatic factors, favouring survival of fungi. Various authors also stated that a greater number of substrates found in certain places can create favourable conditions for fungal growth (Srivoramas *et al*., 2012). In this study, it has been confirmed that households provide good substrates for houseflies and this was also confirmed in a study conducted by Peter *et al*. (2007).

Zarrin *et al*. (2007) also reported a large amount of fungi isolated from external organs of houseflies. The fungal genera isolated from houseflies in this study concur with the results obtained in the work conducted by Zarrin *et al*. (2007) who reported 1295 fungi isolated from the external surface of the flies in Ahwaz, Iran. *Aspergillus* spp., *Penicillium* spp.,Yeasts, *Cladosporium* spp., and *Fusarium* spp., were the frequently isolated. Norberg *et al*. verified the predominance of the genus *Penicillium*. Results from studies conduct by Banjo *et al*. (2002) indicated the presence *Alteruaria* spp., *Fusarium* spp. and *Cladosporium* spp. In another study on isolation and identification of fungi in houseflies, Kaaya and Okech (1990) reported various species isolated, among which *A. flavus*, *A. niger*, *Penicillium* spp. and *Fusarium*. The findings of this study are in agreement with the biology and ecology of houseflies playing an important role as transporters of human diseases (Rajendran and Pandian, 2003). Among the filamentous fungi, *Aspergillus* spp. were isolated in higher percentages from all sample types, an important medical species isolated and has been reported in nosocomial infections (Kontoyiannis and Lewis 2010). Serious life-threatening infections are being reported with an ever increasing array of pathogens, including the well-known opportunists such as *C. albicans* and *A. fumigatus* (Rees *et al*., 1998; Mirza *et al*., 2003; Pfaller *et al*., 2004) though, *A. fumigatus* heads the list of these opportunistic fungi (Denning *et al*., 1998; Lin *et al*., 2001; Diekema *et al*., 2003). *Fusarium* spp. has been reported for rare opportunistic pathogen causing cutaneous and subcutaneous infections, mycotic keratitis, endophthalmitis, osteo-myelitis, and arthritis following traumatic implantation. Additionally, in maize growing areas *F. oxysporum*, *F. solani* and *F. verticillioides*

are common soil fungi, with a world-wide distribution (Zarrin *et al*., 2007; Srivoramas *et al*., 2012). However, in this case it could be possible that houseflies pick-up and spread spores from maize in the field during harvesting, drying and storage. Also, in rural farming communities maize is usually dried in the open thus, resulting in fungal dissemination by houseflies. *Aspergillus niger*, *A. flavus*, *A. parasiticus* were the commonest species isolated from water. Contaminated water may be a route by which fungi are introduced into food when used in food production processes (Hageskal *et al*., 2006; Paterson *et al*., 2009). Thus, this may also explain the high incidence rate of fungal contamination in porridge samples.

In South Africa, it has been observed that there is a lack of knowledge regarding the fungal species and the opportunistic infection that have an association with houseflies. Also, ensuring human health thus requires the control of pathogens such as those transmitted by houseflies (Meerburg *et al*., 2007). Furthermore, to reduce the population of housefly proper hygiene and sanitation practices need to be implemented especially in rural farming communities. On the other hand, the principal methods of control are obvious since the ways in which flies can spread disease are known. Houseflies are of paramount public health concern in areas with poor hygienic and sanitation environments (Nazni *et al*., 2005). In the present study, households and human excreta were identified as common breeding places for houseflies. Successful control of houseflies requires management approach such as proper hygiene practices. Human excreta particularly if known to be infected must be disposed of in such a way that flies cannot possibly reach them. Other sources of infection such as manure and garbage heaps must not be allowed to accumulate for long periods near houses. However, where this cannot be avoided the material should be stored in fly-proof containers. Food for human consumption must always be covered to prevent flies from settling on it. Houseflies could also be controlled effectively by the elimination of breeding habitats (Khan *et al*., 2012; Malik *et al*., 2007). Another approach could be the use of fly traps to reduce the number of adult flies inside buildings and outdoor. This will lead to reduced egg production and larvae (Geden, 2005).

Consequently, there is a serious need to educate rural farmers about the health hazards of flies, poor sanitation practices and how to best manage their farm animal waste to minimize fly breeding. It is crucial to help the people in the study area to comprehend the human health effects

associated with the fungi carried by houseflies and their potential to produce mycotoxins, especially those belonging to the genera of *Aspergillus*, *Penicillium* and *Fusarium* which are important in food commodities. Since, the genera of *Aspergillus*, *Penicillium* and *Fusarium* were isolated from houseflies it therefore implies a potential risk of contamination with aflatoxins, deoxynivalenol, fumonisins, ochratoxin A and zearalenone. Additionally, these mycotoxins can cause a wide range of short-term as well as long-term human health effects, ranging from immediate toxic response to potential long-term carcinogenic and teratogenic effects, particularly in pregnant women (Marasas *et al*., 2004; Missmer *et al*., 2006). Furthermore, given the ever increasing number of individuals at risk from fungal infections, it is imperative that the people in the rural farming community adhere to sustainable housefly control practices. Guaranteeing human health and food safety in the rural communities thus requires the control of fungi and pathogens such as those transmitted by houseflies. This is the first study to clearly demonstrate the potential of houseflies as vectors for the transmission of fungal spores to maize, porridge and water.

3.4 CONCLUSION

There is no need to question the necessity for fly control as houseflies can cause agricultural losses and nuisance. The study has demonstrated the role played by *Musca domestica L.* in disseminating fungal spores and a vector of hazardous diseases. The list of documented fungal pathogens is forever increasing, as a result one can no longer dismiss houseflies as contamination vectors when high incidences of fungal genera are isolated. Furthermore, given the ever increasing number of individuals at risk for fungal infections, it is imperative that the people in the rural farming community in, Gauteng Province adhere to the ways in which houseflies can be controlled. The findings of this study suggest that as researchers we should initiate health education and hygiene promotion programmes for rural farmers with more emphasis on hands-on ways to reduce housefly populations, especially on how to manage common breeding sites conducive for houseflies. However, increasing the awareness of sanitation and knowledge of the hazards associated with houseflies in rural farming communities can play a major role by reducing the spread of houseflies. Another way could be by means of recycling and rebaiting of the fly traps used during sample collection in this study. On the other hand, this method is simple and may be the most effective way to control houseflies and reduce the risk of diseases and

fungal transmission. With the outcome of this method, the community can objectively control housefly population. A range of *Aspergillus*, *Fusarium* and *Penicillium* fungi have been isolated in this study. Of greatest concern is whether the presence of such fungi necessarily determines mycotoxin producing fungi. However, it would be useful to screen for mycotoxigenic potentials and to determine the concentrations in samples, particularly in relation to human exposure.

CHAPTER FOUR

Mycotoxigenic potentials of *Aspergillus*, *Fusarium* and *Penicillium* fungi isolated from houseflies and food commodities in Gauteng Province, South Africa

ABSTRACT

In the previous study, houseflies were shown to dissiminate fungi in food commodoties in Gauteng Provinve, South Africa. The fungi isolated from the houseflies and foodstuffs were used to determine their toxigenic potentials and to quantify the mycotoxin produced. A total of 573 potentially toxigenic isolates of *Aspergillus* (260), *Fusarium* (151) and *Penicillium* (162) species were recovered from housefly, maize, porridge and water that was tested for the production of aflatoxins (AFB_1, AFB_2, AFG_1 and AFG_2), deoxynivalenol (DON), fumonisin B_1 (FB_1) ochratoxin A (OTA) and zearalenone (ZEA) by thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC) respectively. Strains of *A. flavus* and *A. parasiticus* belonging to the genera of *Aspergillus* were found to be the main producers of AFB_1, AFB_2, AFG_1 and AFG_2, while *A. carbonarius*, *A. niger* and *A. ochraceus* produced OTA. From the *Penicillium* genera, production of OTA was observed in strains of *P. verrucosum*. Fumonisin B_1 was produced by *F. verticillioides* and *F. proliferatum* with concentrations ranging from 20-1834 µg/kg and 79-262 µg/kg in isolates from houseflies. However, similar isolates of the same genera were found to produce FB_1 in maize with concentrations ranging from 10-3943 µg/kg and 2-713 µg/kg. Additionally co-production of DON, FB_1 and ZEA toxins was observed in 7% and 14% of the *Fusarium* isolates from houseflies and maize. Deoxynivalenol was the least detected toxin mainly found to be produced by *F. culmorum*, *F. graminearum*, *F. poae* and *F. sporotrichioides*. The high mycotoxins levels produced in isolates from houseflies, maize, porridge and water in this study are regarded as unsafe. Thus, possible human exposure to mycotoxins increases concern with respect to human health and demands consistent investigation.

Key words: Toxigenic fungi, mycotoxins, TLC, HPLC.

4.0 INTRODUCTION

It has become clear that growth of filamentous fungi in food and feedstuffs may results in production of toxins known as mycotoxins (Atanda *et al*., 2013).The consumption of such mycotoxins can cause a variety of health effects in humans (Bhat and Vasanthi, 2003) and their effect upon consumption is equally diverse ranging from acutely toxic to immunosuppressive and carcinogenic (Fadahunsi *et al*., 2011). Although fungi can produce mycotoxins, the production of a particular mycotoxin is restricted to a limited number of fungal species and, in some cases, may be limited to a particular strain within a species (Huwig *et al*., 2001). Many species in the genus *Fusarium*, as well as several *Penicillium* and *Aspergillus* species, are notorious for their ability to produce mycotoxins. *Aspergillus*, *Fusarium* and *Penicillium* are the natural predominant toxigenic fungal genera existing in combination with food commodities (Pitt, 2000c; CAST, 2003). The genus *Fusarium* is destructive on maize crops and other commodities before and immediately after harvest (Abbas *et al*., 2000; Cooney *et al*., 2001). However, certain species of *Aspergillus* and *Penicillium* are also plant pathogens, but these genera are more commonly associated with commodities and foods during drying and storage (Garuba *et al*., 2011). Infection of *Aspergillus*, *Fusarium* and *Penicillium* species is possibly influenced by many factors including, among others, environmental conditions such as the climate, temperature and humidity (Doohan *et al*., 2003; Fandohan *et al*., 2003; Brennan *et al*., 2005; Parsons and Munkvold, 2012). However, in the field and during the storage period, houseflies act as vectors and are often considered as the primary cause of grain losses (Schulthess *et al*., 2002; Majumbar *et al*., 2008).

Although over 400 mycotoxins are known, relatively few are of major concern to human health. Those considered significant are the AFs, OTA and *Fusarium* toxins (DON, FBs and ZEA) (CAST, 2003; WHO, 2006). This is mainly because of their high toxicity and that levels above those currently regulated are often encountered in food and feed commodities. As such, their presence in food commodities and continuous intake especially in under-developed and developing countries are often reported. The consequence of this is that they do not only affect human health, but equally result in serious economic losses. Health effects in humans associated with exposure to mycotoxins are generally termed mycotoxicoses and in severe circumstances, death may result (Voss *et al*., 2002; Glenn *et al*., 2004; Prapagdee *et al*., 2008). Aflatoxins are

produced by certain species *of Aspergillus* where *A. flavus* and *A. parasiticus* are the most economically important (Shundo *et al*., 2009). Aflatoxin B_1 is the most common in food and amongst the most potent genotoxic and carcinogenic aflatoxins. In Kenya, acute aflatoxin poisoning results in liver failure and death in up to 40% of cases (CDC, 2004). Within the genus *Fusarium*, species that produce TH, FB and ZEA mycotoxins have traditionally gained most attention (Goyarts *et al*., 2007; Yazar and Omurtag, 2008). All these groups of mycotoxins can occur together in a range of food commodities, including maize. Deoxynivalenol is a member of the trichothecenes family, the best known common contaminant of grains and their subsequent products. They are produced by many species of fungi, mainly by *F. graminearum* and *F. culmorum* (Sobrova *et al*., 2010). It is also known for its strong emetic effects after ingestion, because it is transported into the brain, where it affects dopaminergic receptors. Furthermore, the emetic effects of this mycotoxin were firstly described in Japanese men consuming mouldy barley containing *Fusarium* fungi in 1972 (Perkowski *et al*., 1990). Fumonisins are common maize contaminants that pose risks to food safety and public health, notably produced by a number of *Fusarium* species: *F. verticillioides, F. proliferatum, F. culmorum, F. avenaceum, F. nivale* and *F. oxysporum* (Marasas, 2001; Pestka and Smolinski, 2005; Soriano *et al*., 2005; Vanara *et al*., 2009). Fumonisin B_1 (FB_1) is the most important fumonisin followed by fumonisin B_2 (FB_2) and fumonisin B_3 (FB_3) (Covarelli *et al*., 2012).

However, the risks result from FB_1 ability to cause diseases, including cancer (Rheeder *et al*., 1992) and neural tube defects (Missmer *et al*., 2006). Another *Fusarium* mycotoxin, ZEA, has been occasionally correlated with hyperoestrogenic syndromes in humans (Zinedine *et al*., 2007) and is produced by a variety of *Fusarium* fungi, including *F. graminearum, F. culmorum, F. equiseti, F. crookwellense* and *F. semitectum,* which are regular contaminants of cereal crops worldwide (Bennett and Klich, 2003). Ochratoxin A is produced by some species of fungi including *Aspergillus* species and *Penicillium verrucosum.* It is nephrotoxic and hepatotoxic and it is likely to have carcinogenic potential in humans (IARC, 1993c). Owing to the various health problems and high economic losses resulting from potentially toxigenic fungi, there is a need to describe the toxigenic fungi associated with various feed and food commodities. And to introduce legislation aimed at limiting and controlling the exposure to these toxic substances. Since toxigenic fungi can be detected in high levels, control of fungal and mycotoxin

contamination has become a priority in the area of research. Although much improvement has been made, relatively little is known about the harmful effects of toxigenic fungi and mycotoxins especially in rural areas of South Africa where there is high risk of fungal contamination. Of concern, *Aspergillus*, *Fusarium* and *Penicillium* species form mycotoxins that are toxic to humans**.** The purpose of this study was therefore to determine the toxigenic potentials of *Aspergillus*, *Fusarium* and *Penicillium* fungi.

4.1 MATERIALS AND METHODS

All reagents used were of analytical grade and the solvents used for HPLC were of HPLC grade obtained from Sigma unless otherwise stated.

(a) Mycotoxin standards

Aflatoxin B_1, B_2, G_1 and G_2, DON, OTA and ZEA standards were obtained from Sigma, St Louis, Mo., USA. Fumonisin B_1 was purchased from PROMEC, MRC, South Africa.

(b) HPLC equipment

HPLC analysis was performed using a Shimadzu system (Shimadzu Corporation, Kyoto, Japan), equipped with liquid chromatograph LC 20A fitted to degasser (DGU 20A3), auto sampler SIL 20A, communications bus module (CBM 20A), column oven (CTO 20A), fluorescence (RF 10AXL), photo diode array detector (SPD M20A) with column Waters Symmetry C18 5 µm, 4.6 x 250 mm, (Waters, Milford, MA, USA) all connected to a Dell computer with Intel Core DUO with Microsoft XP.

4.2 TOXIGENICITY SCREENING

4.2.1 Determination of toxigenic potentials of *Fusarium* species

The ability of isolates to produce toxins were evaluated whereby all isolated *Fusarium* species were plated in Petri dishes containing solid yeast extract sucrose (YES) agar with chloramphenicol and streptomycin to reduce the growth of bacteria, prepared by dissolving 20 g of yeast extract powder + 150 g sucrose + 0.5 g magnesium sulphate + 20 g agar into 885 ml of distilled water and were finally incubated at 25^{o}C for 4 weeks. The culture medium was sterilized in an autoclave at 121^{o}C for 15 mins and further cooled in a water bath to 50^{o}C. *Fusarium* toxins were extracted according to the improved method of Hinojo *et al*. (2005). Ten

grams of macerated agar-containing mycelia was collected into a 250 ml conical flask and 50 ml of acetonitrile (ACN): water (60:40, v/v) was added and placed in a wrist shaker for 1 hour. The entire content was filtered through a Whatman #4 filter paper and the pH was adjusted to 5.9-6.5 with 1M H_2SO_4. The filtrate was then poured into a 250 ml separation funnel and extracted three times with 25 ml dichloromethane (DCM). Twenty-five ml of ACN was added to the solution previously extracted with DCM, filtered through a bed of sodium sulphate anhydrous to remove the moisture. The entire solution was, dried by passing it over a stream of N2 gas with the vial placed on a heating block set at a temperature of 60^{o}C.

4.2.2 Determination of toxigenic potentials of *Aspergillus* and *Penicillium* species

Fungal colonies were individually sub-cultured from Czapek yeast agar (CYA) and malt extract agar (MEA) onto solid yeast extract sucrose (YES). Sub-cultured plates were incubated at 25^{o}C for three weeks after which, mycotoxins were extracted following the agar plug technique according to the method of Bragulat *et al*. (2008) with a slight modification. Briefly, 1 g of agar plug from the fungal colonies including the medium was removed with the aid of a sterile cork borer. The agar plugs were then placed in 4ml amber vials. The toxins from the plugs were extracted with 3 ml of HPLC grade methanol and thoroughly mixed for 1 min using a vortex mixer. After vortexing the extract was filtered through a 0.2 µm syringe filter unit and collected in a screw-cap amber vial. The solution was then evaporated to dryness under a stream of nitrogen gas with the vial placed on a heating block set at a temperature of 60^{o}C. The dried residue was stored at 4^{o}C until further analysed.

4.3 MYCOTOXIN IDENTIFICATION BY TLC and HPLC

A two-dimensional TLC technique was used for detecting Aflatoxins: AFB_1, AFB_2, AFG_1 and AFG_2, DON, FB_1, OTA and ZEA, while HPLC was used quantify the levels of toxins in extracts.

4.3.1 Confirmation of mycotoxins by TLC

An aluminium backed TLC plates (20 x 20 cm) pre-coated with silica gel G (Merck) were lightly pencilled a cross in each corner 15 mm from each edge (These are the two lines at 90^{o}C parallel to two edges, that cross at the origin to assist in the measuring of retardation factor (R_F) values. Four equal 10 x 10 cm plates are guillotined and the crosses became the origin of a two

dimensional chromatograph. The identification of each sample ran was pencilled at each corner diagonally opposite to the origin. The extracts were dissolved with 200 µl of acetonitrile: water (1:1, v/v) and mixed via vortexing. Twenty µl of the extract was spotted onto the origin of the plates in 2 µl portions. The origin was dried at each stage with a steam of warm air using a hot air drier. For the analyses FB_1, 10 ml of [butanol: water: acetic acid (BWA), (12:5:3, v/v)] solvent systems (mobile phase) was prepared and transferred to chromatographic tanks. The tanks were left for about 30 min in order to be saturated by the solvent systems. The plates were inserted into the chromatographic tank with the origin in the bottom left hand corner and allowed the solvent reach the top of the plate. The plates were removed immediately before the solvent over-runs and dried using warm air. The plates were cooled and transferred again into the chromatographic tank at right to the first run, thus the origin was now at the bottom right hand corner. The solvent was allowed to run until the top of the plate, the TLC plates were then removed and allowed to dry and sprayed with anisaldehyde reagent (Merck) prepared by mixing 70 ml methanol (CH_3OH), 5 ml concentrated sulphuric acid (H_2SO_4), 10 ml glacial acetic acid and 0.5 ml p-anisaldehyde (p-methoxy benzaldehyde). The plates were heated briefly for 2 mins at 110^oC. After heating TLC plates with absorbing spots were marked around with a pencil and labelled with a suitable code, e.g. P for purple.

For the analyses of DON, plates were inserted into the chromatographic tank containing mobile phase [dichloromethane: Ethyl-acetate: Propan-2-ol (90:5:5, v/v/v)] for first dimension and [Toluene: ethyl-acetate: formic acid (6:3:1, v/v/v)] for second dimension. The plates were further sprayed with chromotropic acid (CTA) reagent prepared by adding 1 part of 10% CTA solution to 5 parts concentrated sulphuric acid and water (5:3, v/v), heated in an oven at 120^oC for 2 mins and results recorded. A similar procedure was followed for ZEA but in this case, with the mobile phase was [dichloromethane: acetone (90:10, v/v)] and the plates were sprayed with cold diazotised dianisidine reagent. The cold dianisidine reagent was prepared by dissolving 0.5 g of dianisidine in 20 ml of water to which 1.5 ml concentrated hydrochloric acid (HCl) was diluted to 100 ml. This mixture was then added to equal volume of 10% sodium nitrite solution, mixed and cooled to 5^oC. The mixture was allowed to come to room temperature before the plates were sprayed.

For AFB_1, AFB2, AFG_1, AFG_2, and OTA analyses, into the vial containing the dried residues 200 µl of dichloromethane: acetone (1:1, v/v) was added and vortexed for 1 min. Aliquots of 20 µl of extract was spotted about 15 cm above the baseline of the pre-coated silica gel TLC plate (20 x 20 cm). For comparison 20 µl of AFB_1, AFB_2, AFG_1, AFG_2, and OTA standard toxins were also spotted on TLC plate following the similar procedure with those of extracts. The spotted plates were developed two-dimensionally in a TLC developing tank in dichloromethane: ethyl acetate: propan-2-ol (90:5:5, v/v/v) (DEP) following the method of devised by Patterson and Roberts (1979). After the first run through the plate, the plates were immediately removed, dried and transferred into another TLC tank containing 20 ml of toluene:ethyl acetate:formic acid (6:3:1 v/v/v) (TEF).As soon as the developing solvent ran through the plates, the plates were removed and dried with a steam of warm air using a low heat hair-drier including that of the standards. The fluorescing colour of spots produced was observed under long wave UV light at wavelength 365 nm (San Gabriel, USA). The retardation factors (R_F) of the various spots on TLC plates were determined and compared with those of standard toxins (AFB_1, AFB_2, AFG_1, AFG_2, DON, FB_1, OTA and ZEA) to aid in the identification of toxins present. After TLC analyses, all the extracts were dried under a stream of N_2 gas with the vial placed on the heating block set at a temperature of 60^oC and stored at 4^oC for further analysis.

4.3.2 Quantification of mycotoxins by HPLC

After TLC analysis all extracts were made up to 1 000 µl with HPLC grade methanol. Briefly, an aliquot of 250 µl of the extracts were pipetted onto screw-capped HPLC vials. Aflatoxin B_1, AFB_2, AFG_1, AFG_2, DON, FB_1 OTA and ZEA were separated isocratically on HPLC using different mobile phases, wavelength(s) and methods of detection as stipulated below. In addition, in order to avoid inappropriate reporting, species producing toxins obtained from extracts were based on the comparison of the UV spectrum (Aflatoxins and DON), chromatogram of standard (FB_1 [derivatized with o-phthaldialdehyde (OPA) reagent], OTA and ZEA) and the retention times of the detected peaks with those of standard. The minimum detectable amount was for aflatoxins, DON, FB_1, OTA and ZEA analysed from non-producing isolates, whereby three sample replicates were analysed for each isolate were spiked with known concentrations of mycotoxin investigated.

Aflatoxins (Kokkonen *et al*., 2005)
Mobile phase: Water: methanol: acetonitrile: trifluoro-acetic acid (60: 20: 20: 1.25, v/v/v/v)

Detector: Photo-diode array (wavelength: 365 nm)

Flow rate: 1 ml/min

Column heater: 30^{o}C

Deoxynivalenol (Kokkonen *et al*., 2005)
Mobile phase: Water: methanol (85:15, v/v)

Detector: Photo-diode array (wavelength: 220 nm)

Flow rate: 0.4 ml/min

Column heater: 30^{o}C

Fumonisin B_1 (Shephard *et al*., 2000)
Mobile phase: Methanol: sodium di-hydrogen phosphate (80:20, v/v)

Wavelength: 335 nm and 440 nm)

Flow rate: 1 ml/min

Column heater: 40^{o}C

Ochratoxin A (Ghali *et al*., 2009)
Mobile phase: Acetonitrile: methanol: acetic acid (51: 47: 2, v/v/v)

Detector: Fluorescence detector (wavelength: 333 nm and 443 nm)

Flow rate: 0.8 ml/min

Column heater: 40^{o}C

Zearalenone (Abdulkadar *et al*., 2009)
Mobile phase: Acetonitrile: water: methanol (46:46:8, v/v/v)

Detector: Fluorescence (wavelength: 274 nm and 455 nm)

Flow rate: 1 ml/min

Column heater: 40^{o}C

4.3.3 Data analysis

Data analysis was performed to derive mean values, which were compared by least significant difference using all pairwise multiple comparison procedures (Holm-Sidak method) and, a linear regression analysis was conducted on SigmaStat 3.5 for Windows (Systat Inc, 2006).

4.4 RESULTS

4.4.1 Toxigenicity potentials of *Aspergillus*, *Fusarium* and *Penicillium* isolates

A semi-quantitative TLC technique was conducted to confirm the presence of AFs, DON, FB_1, ZEA and OTA toxins. The retardation factors (R_{F1} and R_{F2}) and colour of the individual spots on TLC were circled, calculated and compared with those of standard mycotoxins to assist in the identification of toxins. As indicated all positive extracts for DON, FB_1 and ZEA had spots with R_{F1} and R_{F2} values ranging from 21 and 31 mm, 48 and 58 mm and 80 and 85 mm in Figure 4.1. Of all 85 *Fusarium* isolates from houseflies, highest incidence rate of FB_1 65% (55) was found while; ZEA and DON had the lowest incidence rate of 12% (10) and 5% (4). For *Fusarium* isolates (66) from maize, only 8% (12) 1of the extracts were positive for FB_1 particularly from *F. verticillioides*. The fluorescence of AFs and OTA viewed under ultra violet light revealed that some isolates were positive showing a light blue fluorescence for AFB_1 and AFB_2, light green fluorescence for AFG_1 and AFG_2 as indicated in Figure 4.2, while a blue-green fluorescence was observed for OTA, Fig. 4.3. All positive extracts were recorded with R_F values ranging from 42 and 44 mm for AFB_1, 39 and 41 mm for AFB_2, 37 and 40 mm for AFG_1, 36 and 39 mm for AFG_2, 30 and 32 mm for OTA.

From *Aspergillus* isolates (186) of houseflies, AFs were found with an incidence rate of 11% (20) for AFB_1 and 6% (12) for OTA. While, the *Penicillium* isolates (106) had incidence rates of 16% (17) for OTA. For *Aspergillus* isolates (19) from maize, AFs were recorded with the incidence rate of 58% (11) for AFB_1, 32% (6) for AFB_2 and 16% (3) for AFG_1. However, the *Penicillium* isolates (42) were recorded with the incidence rates of 5% (2) for OTA. Furthermore, AFs in *Aspergillus* isolates (18) from porridge were recorded with incidence rate of 67% (12) for AFB_1, 50% (9) for AFB_2, 6% (1) for AFG_1 and AFG_2, while the *Penicillium* isolates (7) had the incidence rate of 14% (1) for OTA. For *Aspergillus* (37) isolates from water, only 46% (17) fluoresced for AFB_1, 27% (10) for AFB_2, 8% (3) for AFG_1 and 16% (6) for OTA, whereas *Penicillium* isolates (7) had the incidence rate of 14% (1) for OTA. Accordingly, results on TLC confirmed *A. flavus* as main producers of AFB_1 and AFB_2, *A. parasiticus* (AFB_1, AFB_2, AFG_1 and AFG_2), *P. verrucosum* (OTA), *F. verticillioides* and *F. proliferatum* (FB_1) in *Aspergillus*, *Penicillium* and *Fusarium* isolates.

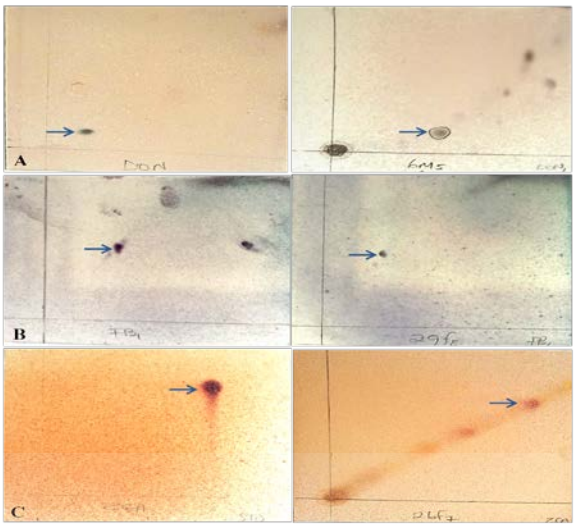


Figure 4.1 View of silica gel coated two-dimensional aluminium baked TLC plates for A: Deoxynivalenol standard (*left*) and Deoxynivalenol produced by *F. graminearum* isolated from houseflies (*right*), B: Fumonisin B_1 standard (*left*) and Fumonisin B_1 produced by *F. verticillioides* isolated from maize (*right*), C: Zearalenone standard (*left*) and Zearalenone produced by *F. oxysporum* isolated from houseflies sample (*left*).

Figure 4.2 View of silica gel coated two-dimensional aluminium baked TLC plates for A: Aflatoxin B_1 (*left*) and B_2 (*right*) standard, B: Aflatoxin B_1 (*left*) and B_2 (*right*) produced by *A. flavus* isolated from houseflies, C: Aflatoxin G_1 (*left*) and G_2 (*right*) standard, D: Aflatoxin G_1 (*left*) and G_2 (*right*) produced by *A. parasiticus* isolated from water sample.

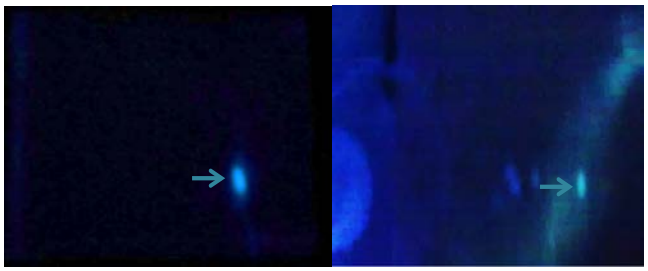


Figure 4.3 View of silica gel coated two-dimensional aluminium baked TLC plates for ochratoxin A standard (*left*) and ochratoxin A produced by *P. verrucosum* isolated from water sample (*right*).

4.4.2 Quantification of toxigenic potentials by HPLC

All the fungal isolates representing the genera of *Aspergillus*, *Fusarium* and *Penicillium* were further quantified for their ability to produce mycotoxins. Results obtained via HPLC analysis were based on the calibration curves, chromatograms of standards and of sample extracts. Figure 4.4 represent the chromatograms of DON produced by *F. culmorum* while those of FB_1 produced by *F. verticillioides* are presented in Fig. 4.5. Zearalenone was among the *Fusarium* toxins detected produced mainly by *F. oxysporum* isolates as chromatograms are presented in Fig. 4.6. Aflatoxin (AFB_1, AFB_2, AFG_1 and AFG_2) was the most mycotoxin detected produced by *A. flavus* and *A. parasiticus*, calibration curve and chromatograms are presented in Fig. 4.7. The calibration curve, chromatograms of standard and extract for the production of OTA by *Penicillium* isolate are presented in Fig. 4.8.

Raw data of the different mycotoxins produced are presented in Appendix II. However, results on the correlation between *Aspergillus*, *Fusarium* and *Penicillium* fungi isolated from houseflies, maize, porridge, water and their toxigenic potentials are summarized in Table 4.1. The total range of concentrations produced from all the fungal isolates are also presented in Table 4.1. Results from the *Aspergillus* isolates revealed that *A. flavus* and *A. parasiticus* were the only two spp. tested positive for production of AFs while, *A. carbonarius*, *A. niger* and *A. ochraceus* were detected for the production of OTA.

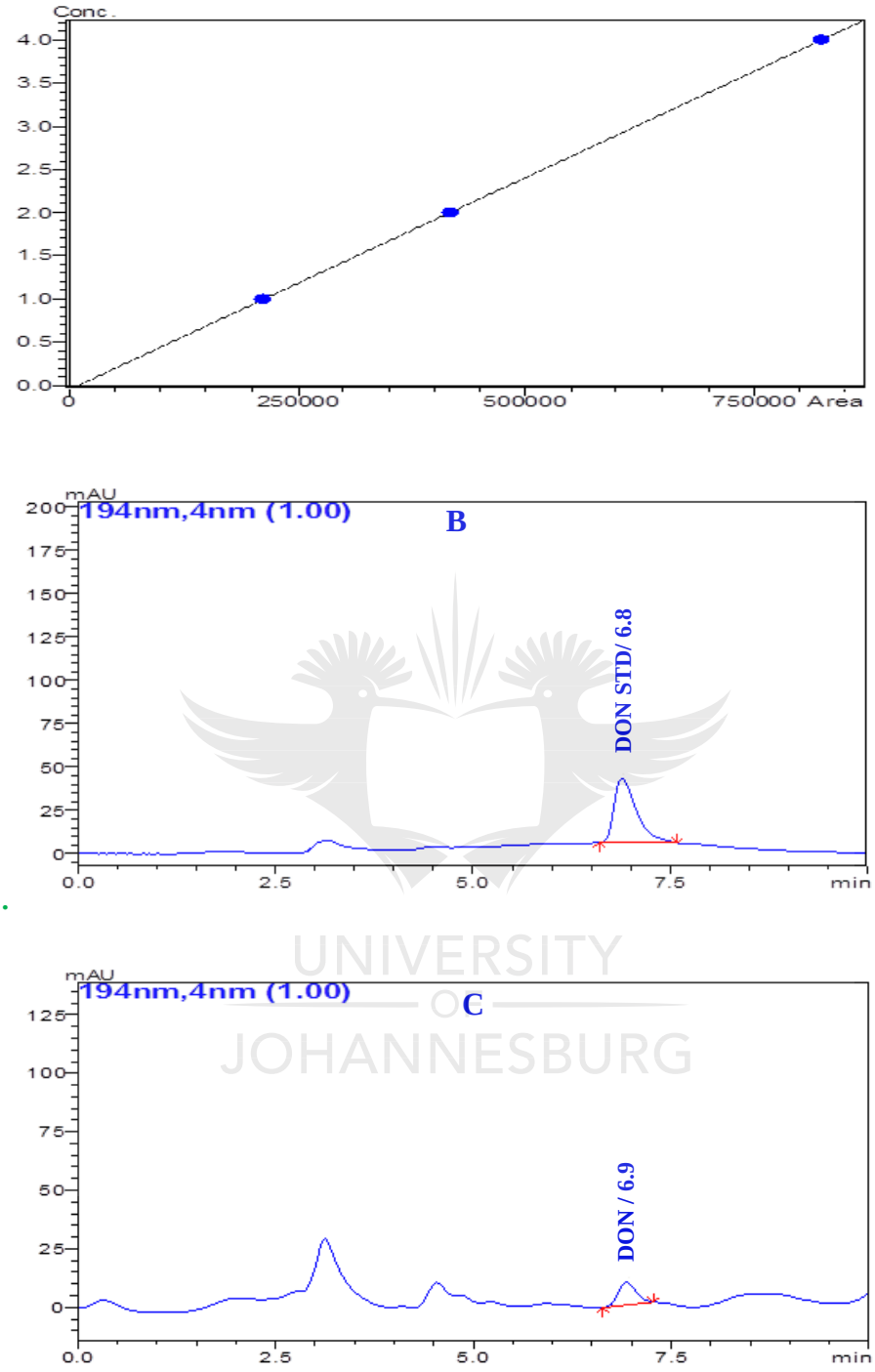


Figure 4.4 Chromatograms of deoxynivalenol. A: Calibration curve of standards at 1, 2 and 4 µg/ml (1 µl injected), correlation coefficients: R^2 = 0.99999); B: Deoxynivalenol standard at retention time of 6.8 min and C: Deoxynivalenol produced by *F. culmorum* (50 µl of extract injected) at retention time of 6.9 min.

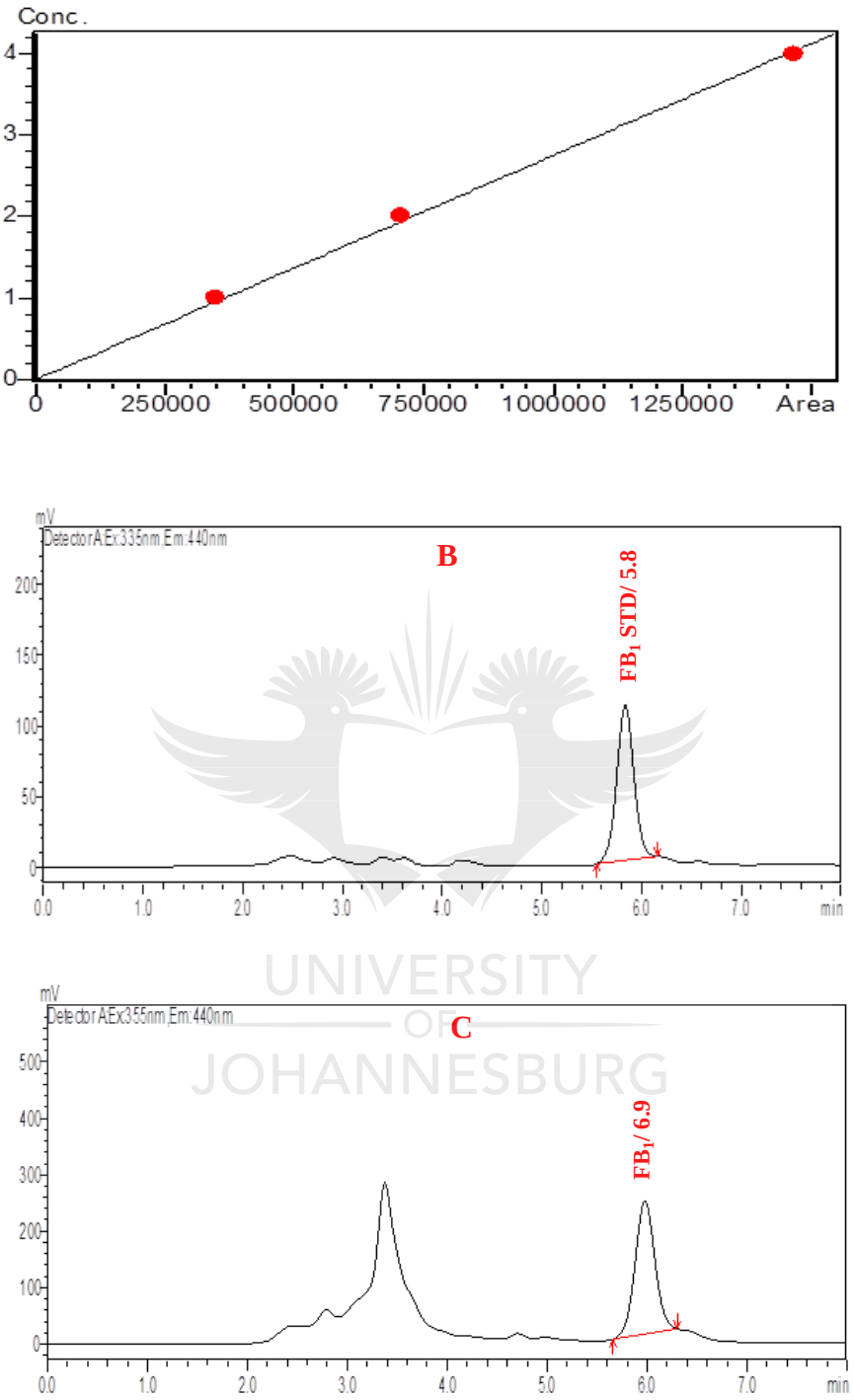


Figure 4.5 Chromatogram of fumonisin B_1. A: Calibration curve standards (1, 2 and 4µg/ml, 1 µl injected), correlation coefficients: R^2 = 0.99997; B: Fumonisin B_1 standard at retention time of 5.8 min and C: Fumonisin B_1 produced by *F. verticillioides* (1 µl of extract injected) at retention time of 5.9 min.

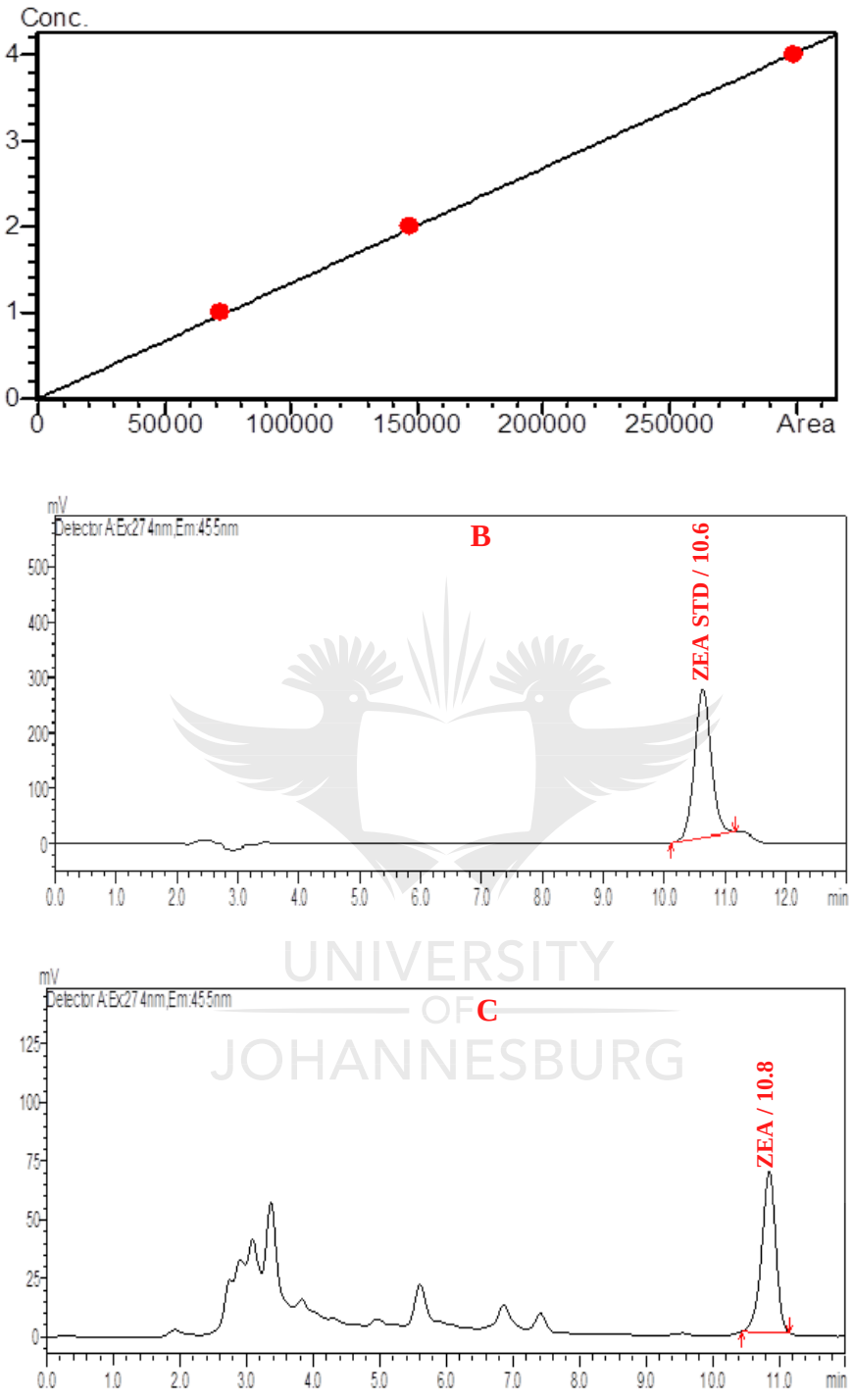


Figure 4.6 Chromatograms of zearalenone. A: Calibration curve of standards (5, 10 and 20 µg/ml, 1 µl injected), correlation coefficients: R^2 = 0.99996; B: Zearalenone standard at retention time of 10.6 min and C: Zearalenone produced by *F. oxysporum* (1 µl of extract injected) at retention time of 10.8 min.

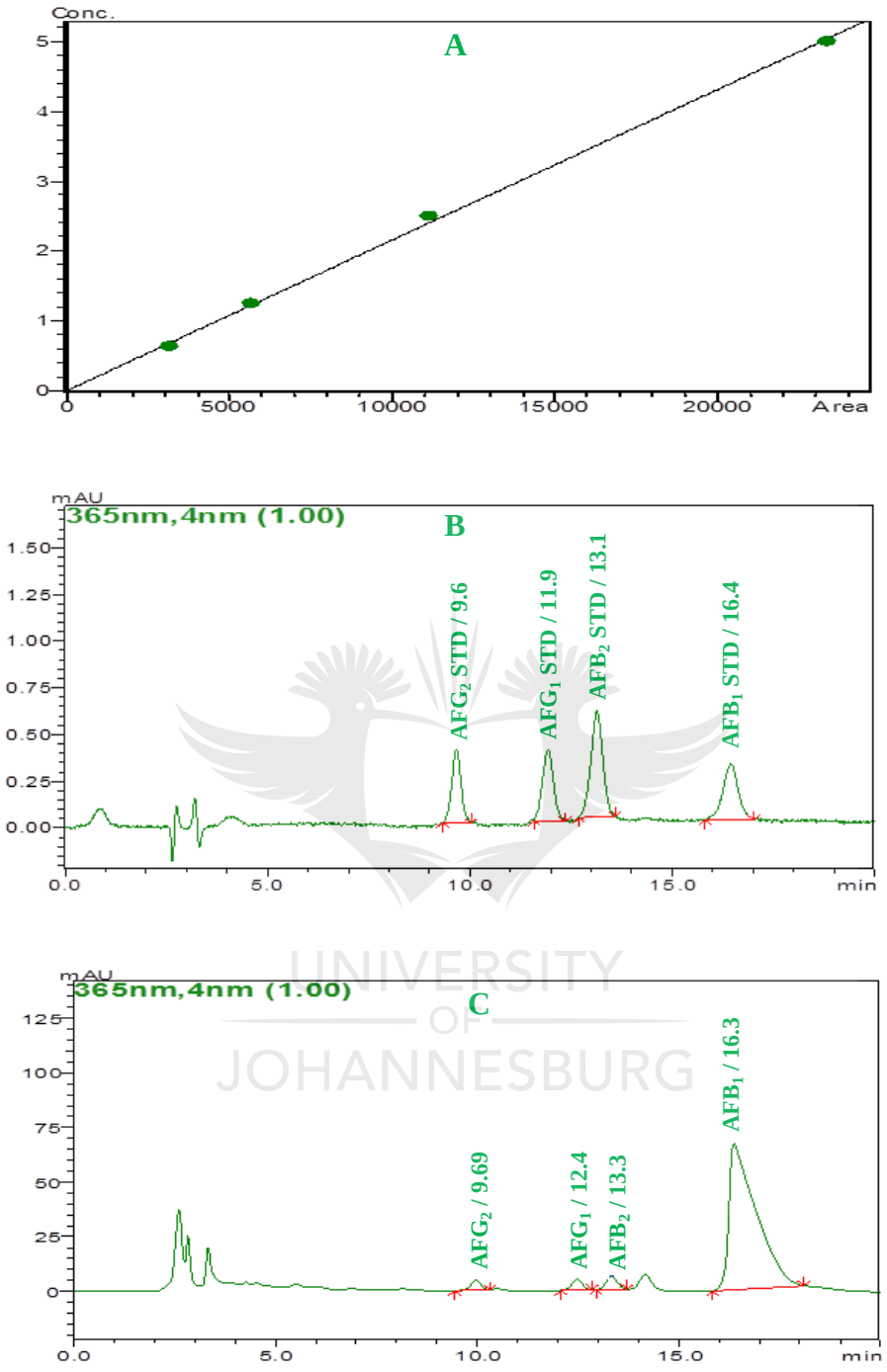


Figure 4.7 Chromatograms of Aflatoxin G_2, G_1, B_2 and B_1. A: Calibration curve of standards at (3 µl injected), correlation coefficients: R^2 = 0.99988; B: Aflatoxin G_2, G_1, B_2 and B_1 standard at retention times of 9.6, 11.9, 13.1 and 16.4 min and C: Aflatoxin G_2, G_1, B_2 and B_1 produced by *A. parasiticus* (10 µl of extract injected) at retention time of 9.9, 12.4, 13.3 and 16.3 min.

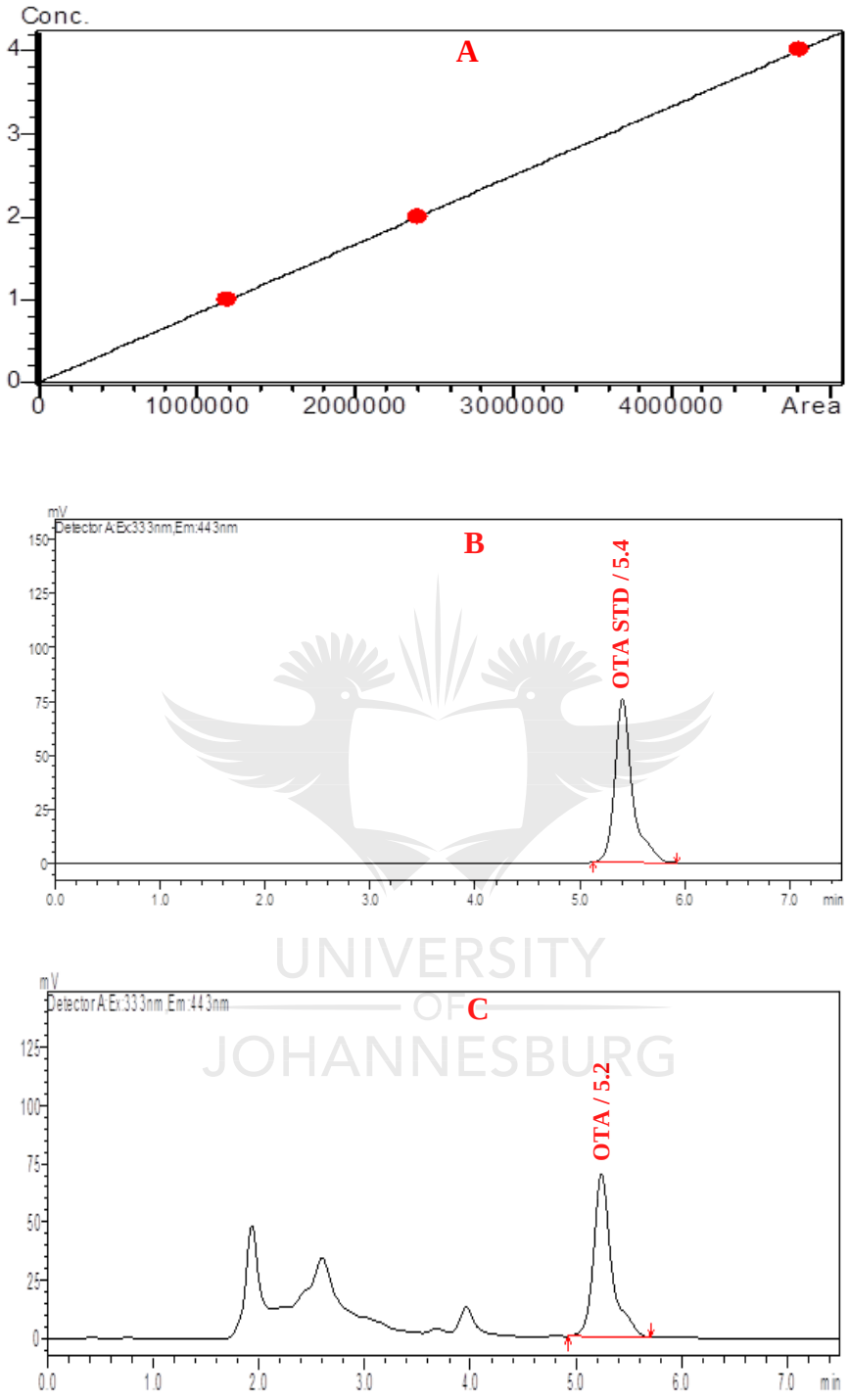


Figure 4.8 Chromatograms of ochratoxin A. A: Calibration curve of standards at 1, 2 and 4 µg/ml (2 µl injected), correlation coefficients: R^2 = 1.00000; B: Ochratoxin A standard at retention time of 5.4 min and C: Ochratoxin A produced by *P. verrucosum* (0.1 µl of extract injected) at retention time of 5.2 min.

As found in the study, AFs production was recorded with the incidence rate of 28% (53/186), 32% (6/19), 39% (7/18), 27% (10/37) and 9% (16/186), 26% (5/19), 28% (5/18), 24% (9/37) produced by *A. flavus* and *A. parasiticus* from houseflies, maize, porridge and water isolates. In addition, the total production of AFs by *A. flavus* was recorded with mean values of 363 µg/kg, 473 µg/kg, 2134 µg/kg and 1620 µg/kg of aflotoxin B_1 and B_2 toxins for houseflies, maize, porridge and water isolates. Whilst, those produced by *A. parasiticus* were detected with AFB_1, AFB_2, $AFG_1$and AFG_2 ranging from 239 µg/kg, 1809 µg/kg, 2089 µg/kg and 2293 µg/kg. The prevalence and mean concentrations were also recorded for OTA produced by some of the *Aspergillus* spp. mainly by *A. niger* 93% (273 µg/kg), 100% (927 µg/kg, 25 µg/kg and 573 µg/kg) from houseflies, maize, porridge and water; *A. carbonarius* 100% (253 µg/kg,1973 µg/kg and 17 µg/kg); *A. ochraceus* 88% (581 µg/kg), 100% (2810 µg/kg and 6 µg/kg) from houseflies, maize and porridge isolates (Table 4.1). *Penicillium verrucosum* was the only isolate among the *Penicillium* spp. recorded with mean concentrations of 855 µg/kg, 10 µg/kg, 60 µg/kg and 8µg/kg for OTA production from houseflies, maize, porridge and water (Table 4.1).

Amongst the *Fusarium* spp., FB_1 was the most common mycotoxin produced by, *F. verticillioides* and *F. proliferatum* with incidence rate of 89 % and 96 %, 100% in isolates from houseflies and maize. However, the highest mean concentration of 666 µg/kg was produced by *F. verticillioides* from maize, while the least mean concentration of 413 µg/kg was produced from houseflies isolates. In addition to *Fusarium* mycotoxins, ZEA was produced by similar species from both houseflies and maize isolates, namely *F. culmorum*, *F. equiseti*, *F. graminearum*, *F. oxysporum* and *F. semitectum* (Table 4.1). Although these species were found to produce ZEA, the incidence rates were observed at 100% (9/9) and 90% (9/10) from *F. culmorum*, 100% (2/2) from *F. equiseti*, 100% (3/3) and (7/7) from *F. graminearum*, 100% (12/12) and 91% (10/11) from *F. oxysporum*, 100% (3/3) and 67% (2/3) from *F. semitectum* in isolates from houseflies and maize. The mean concentrations were recorded to be 273 µg/kg and 5 µg/kg (*F. culmorum*), 63 µg/kg (*F. equiseti*), 798 µg/kg and 8 µg/kg (*F. graminearum*), 108 µg/kg and 6 µg/kg (*F. oxysporum*), 80 µg/kg and 5 µg/kg (*F. semitectum*) from houseflies and maize isolates. Furthermore, DON was the least toxin produced by *Fusarium* isolates including *F. culmorum*, *F. graminearum*, *F. poae* and *F. sporotrichioides*, with an exception to *F. poae* which was only from houseflies (Table 4.1).

Table 4.1 Production of mycotoxins by *Aspergillus*, *Fusarium* and *Penicillium* species isolated from houseflies, maize, porridge and water samples from Gauteng Province, South Africa.

Fungal sources	Fungal species	Number of toxigenic species[#]	Toxin produced	Range of total toxin produced*
HOUSEFLY	***Aspergillus* species (186)**			
	A. candidus	1 (0)	-	-
	A. carbonarius	17 (17)	OTA	38-701
	A. clavatus	6 (0)	-	-
	A. flavus	68 (52, 36)	AFB_1, AFB_2	4-5016, 4-149
	A. fumigatus	12 (0)	-	-
	A. niger	29 (27)	OTA	18-1642
	A. ochraceus	17 (15)	OTA	33-2561
	A. oryzae	3 (0)	-	-
	A. parasiticus	16 (16, 11, 6, 2)	AFB_1, AFB_2, AFG_1, AFG_2	4-1562, 4-146, 3-113, 4-49
	A. ustus	10 (0)	-	-
	A. wentii	2 (0)	-	-
	***Fusarium* species (85)**			
	F. avenaceum	4 (0)	-	-
	F. culmorum	9 (5, 9)	DON, ZEA	2-6, 1-1263
	F. equiseti	2 (2)	ZEA	8-117
	F. graminearum	3 (3)	DON, ZEA	1-4, 2-758
	F. nivale	2 (0)	-	-
	F. oxysporum	12 (12)	ZEA	2-918
	F. poae	5 (4)	DON	1-3
	F. proliferatum	18 (16)	FB_1	84-249
	F. semitectum	3 (3)	ZEA	30-80
	F. sporotrichioides	3 (2)	DON	2-3
	F. verticillioides	24 (23)	FB_1	12-1816
	***Penicillium* species (106)**			
	P. aurantiogriseum	22 (0)	-	-
	P. brevicompactum	17 (0)	-	-
	P. citrinum	5 (0)	-	-
	P. crustosum	12 (0)	-	-
	P. expansum	1 (0)	-	-
	P. janthinellum	15 (0)	-	-
	P. oslonii	10 (0)	-	-
	P. sclerotiorum	1 (0)	-	-
	P. verrucosum	23 (23)	OTA	29-1994
MAIZE	***Aspergillus* species (19)**			
	A. carbonarius	4 (4)	OTA	669-2945
	A. flavus	6 (6, 5)	AFB_1, AFB_2	2120-17988, 74-5025
	A. niger	3 (3)	OTA	4-1518
	A. ochraceus	1 (1)	OTA	2810
	A. parasiticus	5 (5, 5, 3, 0)	AFB_1, AFB_2, AFG_1, AFG_2	55-8541, 47-3047, 107-735
	***Fusarium* species (66)**			
	F. avenaceum	4 (4)	-	-
	F. culmorum	10 (9)	DON, ZEA	1-8, 2-11
	F. graminearum	7 (6, 7)	DON, ZEA	1-5, 1-19
	F. nivale	6 (0)	-	-
	F. oxysporum	11 (10)	ZEA	1-16
	F. proliferatum	7 (7)	FB_1	3-236
	F. semitectum	3 (2)	ZEA	3-6
	F. sporotrichioides	3 (3)	DON, ZEA	3-8
	F. verticillioides	15 (15)	FB_1	7-3920

Note: AFB_1= Aflatoxin B_1, AFB_2= Aflatoxin B_2, AFG_1= Aflatoxin G_1, AFG_2= Aflatoxin G_2, DON= Deoxynivalenol, FB_1= Fumonisin B_1, OTA= Ochratoxin A, ZEA= Zearalenone.*Range of concentration for mycotoxins produced are presented in ug/kg. [#]Number of positive isolates from total colonies isolated in bracket.

Table 4.1 Continued……

Fungal sources	Fungal species	Number of toxigenic species[#]	Toxin produced	Range of total toxin produced*
MAIZE	***Penicillium* species (42)**			
	P. aurantiogriseum	5 (0)	-	-
	P. brevicompactum	3 (0)	-	-
	P. citrinum	7 (0)	-	-
	P. crustosum	1 (0)	-	-
	P. expansum	5 (0)	-	-
	P. janthinellum	5 (0)	-	-
	P. oslonii	12 (0)	-	-
	P. verrucosum	5 (2)	OTA	6-13
PORRIDGE	***Aspergillus* species (18)**			
	A. carbonarius	1 (1)	OTA	17
	A. flavus	7 (7, 5)	AFB_1, AFB_2	1068-5294, 201-4911
	A. niger	4 (4)	OTA	0.3-50
	A. ochraceus	1 (1)	OTA	6
	A. parasiticus	5 (5, 5, 2, 1)	AFB_1, AFB_2, AFG_1, AFG_2	2168, 632-2587, 489-895, 798
	***Penicillium* species (7)**			
	P. aurantiogriseum	1(0)	-	-
	P. citrinum	1(0)	-	-
	P. janthinellum	1(0)	-	-
	P. oslonii	2(0)	-	-
	P. verrucosum	2 (1)	OTA	0-60
WATER	***Aspergillus* species (37)**			
	A. flavus	10 (10, 9)	AFB_1, AFB_2	148-5713, 128-2108
	A. niger	18 (18)	OTA	0.3-5000
	A. parasiticus	9 (9, 7, 5, 1)	AFB_1, AFB_2, AFG_1, AFG_2	329-7974, 359-7642, 131-1195, 101
	***Penicillium* species (7)**			
	P. oslonii	5 (0)	-	-
	P. verrucosum	2 (1)	OTA	8

Note: AFB_1= Aflatoxin B_1, AFB_2= Aflatoxin B_2, AFG_1= Aflatoxin G_1, AFG_2= Aflatoxin G_2, DON= Deoxynivalenol, FB_1= Fumonisin B_1, OTA= Ochratoxin A, ZEA= Zearalenone.*Range of concentration for mycotoxins produced are presented in ug/kg. [#]Number of positive isolates from total colonies isolated in bracket.

4.5 DISCUSSION

The correlation of mycotoxins produced from *Aspergillus*, *Fusarium* and *Penicillium* species isolated from houseflies, maize, porridge and water were found in this study. However, the results revealed that there are many toxigenic species recovered from the isolates that were producers of AFB_1, AFB_2, AFG_1 and AFG_2, DON, FB_1, OTA and ZEA under laboratory conditions. Furthermore in the study, the toxigenic potentials in some of the isolates were not observed, however, detected in some. This could be due to that the distribution and development of *Aspergillus*, *Fusarium* and *Penicillium* fungi and mycotoxin production are determined by unfavourable conditions such as humidity, nutrients and temperature (Doohan *et al*., 2003;

Schaafsma and Hooker, 2007; Shwab and Keller, 2008). Such favourable conditions may influence the growth, survival, distribution of toxigenic fungi. Various authors have extensively reviewed the influence of climatic factors with respect toxigenic potential of *Aspergillus*, *Fusarium* and *Penicillium* fungi (Miedaner *et al*., 2001; Magg *et al*., 2002). Additionally, secondary metabolism of fungi is measured by genetic mechanisms which react to different stimuli from the environment. Also, it is not known exactly how all these factors act and are regulated at the molecular level during the toxin production. The same isolate can produce a wide range of mycotoxins under different culture media (Kostecki *et al*., 1999; Velluti *et al*., 2000; 2001; Vogelgsang *et al*., 2008). On the other hand, fungal isolates may vary significantly in their capability to produce mycotoxins. However not all fungal species possess the genes for the production of certain metabolites (Kokkenen *et al*., 2010). Differences between the AFs produced from all four sample types were noted with *A. flavus* and *A. parasiticus* being the most prevalent AFs producers. In addition, both the B (AFB_1 and AFB_2) and G types (AFG_1 and AFG_2) were produced by *A. parasiticus* while the B types were mainly produced by *A. flavus* (raw data shown in Appendix II). Despite the ability of AFs production by several genera of *Aspergillus* in this study, similar reports on AFs production by some members of *Aspergillus* group are presented by CAST (2003); Somashekar *et al*. (2004); Frisvad *et al*., 2005. Although a few *A. ochraceus* strains were isolated, results indicate that the isolates were able to produce OTA together with other species belonging to the *Aspergillus* group such as *A. niger* and *A. carbonarius*. The production of OTA by *A. carbonarius, A. niger* and *A. ochraceus* have been confirmed (Abarca *et al*., 2003; Mitchell *et al*., 2003; Belli *et al*., 2004; Juan *et al*., 2007) and reported in literature by Klich (2002a).

Fusarium isolates from houseflies and maize were able to produce more than one *Fusarium* mycotoxin. The *Fusarium* mycotoxins produced at high frequencies was FB_1 followed by ZEA particularly by *F. verticillioides* and *F. proliferatum* for FB_1, *F. culmorum* and *F. oxysporum* for ZEA, suggesting that these isolates could the implicated in the possible head blight in maize kernels in South Africa (Fandohan *et al*., 2003; Boutigny *et al*., 2011a; Boutigny *et al*., 2011b). On the other hand, FB_1 and ZEA could be found in the same commodities as secondary metabolites of some toxigenic fungi of *Fusarium* genera. Furthermore, in the study DON was the least mycotoxin produced from *F. culmorum*, *F. graminearum*, *F. poae* and *F. sporotrichioides*

fungi. Despite the less frequency of DON production from *F. graminearum* than *F. poae* and *F. culmorum* isolates, this could be attributed to the lower competitive capacity of *F.* g*raminearum* in the presence of other *Fusarium* species (Muthomi *et al.*, 2008) and that the environmental conditions favoured the germination of fungal spores for *F. poae*, and *F. culmorum* than *F. graminearum*. It is also possible that, due to competition among species these species were stimulating DON production in *F. poae* and *F. culmorum* (Thrane *et al*., 2004).

Moreover, it was observed that *F. culmorum* and *F. graminearum* co-produced DON and ZEA from houseflies and maize isolates. The results showed differences in the frequency of the different *Fusarium* species and in the relevant mycotoxins produced. In the literature, DON and ZEA have been described as the main secondary metabolites of *F. graminearum* and *F. culmorum* (Langseth *et al*., 1998, 2001). According to Marasas (1991), *F. equiseti*, *F. graminearum*, *F. poae*, *F. verticillioides* and *F. sporotrichioides* are considered the most toxic *Fusarium* species while *F. graminearum* is considered the most important producer of DON and ZEA. In this study, other isolates such as *F. semitectum* and *F. sporotrichioides* were also found to produce DON and ZEA. Nevertheless, these findings are in accordance with the data reported by (Ross *et al*., 2001) which revealed that *F. semitectum* and *F. sporotrichioides* produce fungal spores under humid conditions. However, further research is needed since less is known about the co-occurrence of *Fusarium* toxins and on the toxigenicity of fungal spores from houseflies. These results give valuable information on the toxigenicity of some important *Fusarium* species isolated from maize in correlation to those on the external surfaces of houseflies. A wide variety of fungal species from houseflies, maize, porridge and drinking water have been found to produce mycotoxins in this study. Mycotoxins such as AFs, OTA, DON, FB_1, and ZEA produced in this study have been reported to cause health problems in humans. As found, some fungi were capable of producing more than one mycotoxin and some mycotoxins are produced by more than one fungal species. On the other hand, these mycotoxins may also lead to adverse effects to the health of the rural population especially of the fact that populations of developing countries are the most susceptible to diseases caused by mycotoxins (Zain, 2011). Furthermore, it has been reported that people in developing countries worldwide are at risk of chronic exposure to these mycotoxins through contaminated foods (Shephard, 2006; Williams *et al*., 2004). Consumption of maize, food and water containing mycotoxins may lead to development of

serious ailments, such as alimentary toxic aleukia (Al-Gabr *et al*., 2013). Recently, several researchers have reported mycotoxins in maize and maize-based products but the reports on mycotoxins produced here in houseflies and drinking water in South Africa is scanty. Additionally, this is the first study in South Africa to investigate the toxigenic potentials of *Aspergillus*, *Penicillium* and *Fusarium* isolates from houseflies, maize, porridge and water.

4.6 CONCLUSION

There was a correlation observed between the fungal genera of *Aspergillus*, *Penicillium* and *Fusarium* from houseflies, maize, porridge and water samples and the levels of mycotoxins produced. Thus, it indicates that the presence of *Aspergillus*, *Penicillium* and *Fusarium* isolates in this study indicate mycotoxin producing fungi. Although the number of *Aspergillus*, *Fusarium* and *Penicillium* contamination in food commodities is common worldwide, the concentrations of mycotoxins produced by some of the isolates were rather high in this study. This could be explained by the fact that there may be existence of factors enhancing the synthesis of mycotoxins in the medium. The high levels of mycotoxins produced from many of the isolates raise matters of serious concern regarding the spread of toxigenic fungi in human food commodities. Furthermore, the toxigenicity of multiple mycotoxins by a single organism or by a mixture of fungi within the same laboratory conditions in this study presents a problem that has not been sufficiently investigated. Such co-existence of toxins as well as the correlations between various mycotoxins may have additional and synergistic effects in humans that need to be further investigated. It is worth noting the fact that the much higher incidence of co-production, particularly among toxigenic genera of the *Aspergillus*, *Penicillium* and *Fusarium* has been reported in the present study, is a cause for concern. Furthermore, the high levels of mycotoxins may impose negative impact on human health. As a result there is a need to further investigate the toxicity effects of these levels of mycotoxins produced in this study on human lymphocytes.

CHAPTER FIVE

Cytotoxicity effects of *Aspergillus*, *Fusarium* and *Penicillium* extracts on human mononuclear cells

ABSTRACT

People are exposed to different mycotoxins that may have a negative impact on their health. Due to the toxicity and their effects on human, the study was designed to assess cytotoxic effects against human mononuclear cells using methyl thiazol tetrazolium assay; as a means of detecting mycotoxins derived from houseflies, maize, porridge and water extracts. The PHA- stimulated mononuclear cells were exposed to 20, 40 and 80 µ1/ml of mycotoxins containing extracts over 24, 48 and 72 hrs incubation period. The results showed that FB_1 induced the highest decrease in cell viability as compared to DON and ZEA when exposed individually, while exposure to combined mycotoxins considerably showed further reduction of cell viability. Furthermore, cytotoxicity effect of AFs and OTA increased simultaneously with the increase of the mycotoxin concentration from 40 to 80 µl/ml and with the prolongation of exposure time at 48 and 72 hrs. Mononuclear cells treated with AFs+OTA showed greater effect than cells treated with AFs and OTA alone. The results indicated that PHA-stimulated human mononuclear cells were sensitive to mycotoxins containing extracts, as indicated on the decreased cell viability upon exposure to the toxic compounds. However, this activity increased with increase concentrations and duration of mycotoxin exposure to mononuclear cells. In conclusion, the effects of *Aspergillus*, *Fusarium* and *Penicillium* extracts obtained from cultures isolated from houseflies, maize, porridge and water were capable of decreasing cell viability of human lymphocytes within 24, 48 and 72 hrs of exposure.

Key words: Cytotoxicity, mycotoxins, human lymphocytes, methylthiozol tetrazolium assay

5.0 INTRODUCTION

Fungal isolates belonging to the genera of *Aspergillus*, *Fusarium* and *Penicillium* as mycotoxin producing fungi and their possible contribution to health hazards after consumption cannot be overestimated. Deoxynivalenol, FB_1, ZEA, AFs and OTA are amongst the most important mycotoxins produced by these fungal genera (in Chapter 4) with FB_1 being prominent in maize which is a major crop in South Africa consumed by many black Africans, as a staple (Dutton, 2009). The health problems associated with secondary fungal metabolites are readily understood when it is appreciated that over 300 of mycotoxins exist and a vast majority of which have not been confirmed for toxicity (WHO, 2006). In Gauteng Province of South Africa, it is likely that the population is exposed to a diet that contains high levels of mycotoxin and in many cases; there may be other toxins present. For example, in Chapter four DON, FB_1 and ZEA were produced simultaneously by *Fusarium* fungi, while AFs and OTA were produced by *Aspergillus* and *Penicillium* fungi. Co-existence of mycotoxins is of great concern, particularly in the case of FBs (a cancer promoter) and AFs (a human carcinogen) where a complimentary and sometimes synergistic toxicity mechanism of action occurs. A biological effect of mycotoxin consumption by human is the generation of mycotoxin-induced apoptosis which has been observed (Maenetje *et al*., 2008; Makun *et al*., 2011). However, *in vivo* exposures to mycotoxins must take into account individual variances such as the uptake, metabolism, the duration or dose of exposure of the toxins and other factors for instance nutritional status, as well as the biological state of the affected individuals. Whilst *in vitro* exposures can reveal direct synergistic effects of some mycotoxin producing fungi. Cytotoxicity testing using human mononuclear cells by MTT assay (Meky *et al*., 2001) is amongst the methods that can be used to assess the levels of toxicity in human foods and feeds.

Among these mycotoxins, AFs have been the subject of intensive research because they show an emphasized carcinogenic effect on their consumers (Hoogenboom *et al*., 2001; Wild and Gong, 2010). After the invasion of AFs into the liver, lipids infiltrate hepatocytes and this result in liver cell death (Hussein and Brasel, 2001). The mechanism contributing to cell necrosis is the reaction of aflatoxin metabolites with different cell proteins, which leads to inhibition of carbohydrate and lipid metabolism and protein synthesis (Fink-Grernmels, 1999; Wild and Turner, 2002). Epidemiological studies proved that consumption of aflatoxin B_1 contributes

significantly to the high incidence of human liver cancer especially in many developing countries (Henry *et al*., 1999). Several outbreaks of human AFs poisoning have occurred mostly among adults in rural populations with a deprived level of nutrition for whom maize is the staple diet (Lewis *et al*., 2005). Additionally, studies in Gambian children and adults demonstrated a strong link between AFs exposure and reduced immune-competence suggesting that aflatoxin consumption reduces resistance to infection in human populations (Turner *et al*., 2003; Jiang *et al*., 2005). Sharma (2004) reported that hepatocellular carcinoma is predominant in various parts of the world where high levels of AFs in the diet have been documented.

On the other hand, experiments in South Africa showed that FB_1 is the most occurring of the known fumonisins and has been linked to acute diseases (Tseng and Liu, 2001), particularly in rural black populations where maize and maize-based products are consumed on a daily basis (Dutton, 2009). According to Lino *et al*. (2007), neural tube defect in infants is associated to intake of maize-contaminated by fumonisin. Furthermore, when FB_1 is consumed it causes sphinganine and sphingosine accumulation and cell membrane dysfunction. Sphingoid free bases in return, functions as cancer promoters and mutations (Soriano *et al*., 2005; Ghiasian *et al*., 2009; Roohi *et al*., 2012). Zearalenone a hazardously oestrogenic mycotoxin, its toxic effects and metabolites have been ascribed primarily to its chemical structure that resembles that of naturally occurring oestrogens (Gromadzka *et al*., 2008). Although the association between zearalenone exposure and human diseases remains speculative at present, evidence from Puerto Rico suggested that ZEA is correlated with outbreaks of precocious sexual development in thousands of young children exposed to contaminated food. It is assumed that exposure occurred during pregnancy of their mothers who consumed contaminated food during the time of pregnancy. Furthermore, ZEA have been suggested to have a possible link in human cervical cancer and premature initial breast development (Zinedine *et al*., 2007). In South America there have been suggestions that ZEA is the cause for premature menarche in young girls nevertheless these reports have not been proven (Hagler *et al*., 2001).

Several studies have been conducted in a range of cell lines to investigate the cytotoxic effects of DON and other mycotoxins (Meky *et al*., 2001; Pavlina *et al*., 2010). Outbreaks and potential impact of DON may occur after consumption of contaminated food products (Pestka and Zhou,

2000). Deoxynivalenol affects human health causing decrease growth and feed consumption at low concentrations in the diet whereas it induces vomiting at higher acute doses (Flannery *et al*., 2011). Nevertheless, since DON is commonly detected in maize-based foods, there are significant questions regarding the risks of acute poisoning and chronic effects posed to persons ingesting this toxin (Pestka, 2010). Ochratoxins are potent toxins and their presence in food is detrimental. The most significant and toxic ochratoxin is OTA and it contribute to nephrotoxicity and carcinogenicity in humans (Reddy and Bhoola, 2010). Further, studies on dietary intake have confirmed link between endemic nephrotoxicity in humans to their daily domestic ingestion of OTA (Petkova-Bocharova *et al*., 2003; Fuchs and Peraica, 2005; Walker and Larsen, 2005).

In *vitro* toxicity tests are cost-effective and fast way to screen for the toxic effects of mycotoxins because they exclude the need to use laboratory animals and facilitate the assessment of many extracts under similar test conditions. In cytotoxicity tests, the viability of the cells is tested in the presence of possible toxicants. The results of the test, in general, can be based on the ability of viable cells to reduce methyl tetrazolium bromide to a purple formazan dye that can be quantitated by means of microplate spectrophometry and therefore give a degree of the cytotoxicity of added mycotoxins (Jestoi *et al*., 2004). Consequently the distribution of mycotoxin producing fungi by houseflies, maize, porridge and water toward human has not been fully assessed. However, a long-term exposure to high levels of these mycotoxins by the public could be a major concern. Because little information is known regarding the potential cytotoxic effect of mycotoxins on human cells in Gauteng Province, the cytotoxicity effects of *Aspergillus*, *Fusarium* and *Penicillium* genera were investigated on isolated human lymphocytes in extracts derived from houseflies, maize, porridge and water samples.

5.1 MATERIALS AND METHODS

5.1.1 Mycotoxin standards and chemicals

All chemical used were of analytical grade and unless otherwise stated. Roswell Park Memorial Institute (RPMI)-1640 medium (with L-glutamine), complete culture media (CCM), Histopaque-1119, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), phosphate buffered saline (PBS)- pH 7.4, dimethyl sulphoxide (DMSO), foetal bovine serum (FBS), penicillin/streptomycin (Penstrep), phyto-haemagglutinin-p (PHA) and Trypan blue were purchased from Sigma, St Louis, Mo., USA.

5.1.2 Isolation, identification and quantification of fungal extracts analysis by HPLC

Isolation and identification of fungi were conducted according to the method described in Chapter 3 in section 3.1.5 and 3.1.6. Fungal extraction and quantification of extracts by HPLC were conducted following the method described in Chapter 4 in section 4.2.1 and 4.2.2.

5.1.3 Preparation of Methyl thiazol tetrazolium salt (MTT)

Methyl thiazol tetrazolium salt (5 mg) was dissolved in 1ml of 0.14 M PBS to give a concentration of 5 mg/ml. The suspension was through a 0.22 μm pore size syringe filter to sterilize. The sterile stock solution of MTT can be stored in the dark at 4°C until use.

5.1.4 Isolation and purification of lymphocytes

Ethical clearance to undertake this study was obtained from the ethical committee of Faculty of Health Sciences, University of Johannesburg. Purification and isolation of mononuclear cells were conducted according to Meky *et al*., 2001. Blood (5x 6 ml) was collected from a healthy donor into 10 ml heparin tubes and mixed with an equal volume of CCM (RPMI-1640 medium, supplemented with 10% Foetal calf/bovine serum (FBS), 100U/ml penicillin and 100μg/ml streptomycin - Penstrep). The mixture was gently overlaid on equal quantity of Histopaque-1119 using centrifuge tubes (15 ml) and centrifuge at 3000 rpm for 30 min. The interface layer (middle layer between the plasma and Histopaque) consisting of mononuclear cells was carefully collected with a sterile pipette. The cells were washed twice with an equal quantity of RPMI-1640 medium and each time centrifuged at 3000 rpm for 5 min discarding the supernatant. After the second washing, cells (between 1 x 10^5 and 1x 10^6) were gently re-suspended in CCM to give the desired concentration cells/ml.

5.1.5 Enumeration of Cells by trypan blue staining Using Neubauer Haemocytometer

The concentrations of cells were examined as the cells were mixed with 0.2 % trypan blue (dilution factor 5) and counting via a Neubauer haemocytometer using the following procedure: whereby 10 μl of cell suspension was mixed with 40 μl 0.2 % trypan blue, and transferred 10 μl of the same mixture to both chambers of Neubauer haemocytometer covered with a glass cover slip. The number of cells in 1 ml can be calculated using the formula:

n / v × dilution factor (5) × 10^4.

Whereas: n = number of cells counted in all squares

v = number of the squares (with 0,1 µl volume - 1/1/0,1 mm) counted.

The percentage of cell viability was calculated via the formula:

% cell viability = viable cells (unstained) / total number of cells × 100.

5.1.6 Experimental Design

To prepare for the assay, *Aspergillus*, *Fusarium* and *Penicillium* extracts from houseflies, maize, porridge and water samples were re-dissolved in RPMI-1640. The extracts were randomly selected based on those with high, medium and low concentrations on HPLC. Cells having 98% viability were transferred into a cell culture flask containing 100 ml CCM, gently mixed and incubated for 24 hrs at 37^{o}C in a 5% CO_2-buffered and humidified incubator. After incubation period, 20 µl of the human mononuclear cells was pipetted in a 96-well culture plates in triplicates. To the wells in triplicate 20 µl phytohaemagglutinin (PHA 10 µg/ml) was added as a stimulant. The cells were exposed to *Aspergillus*, *Fusarium* and *Penicillium* extracts at various concentration levels of 20, 40 and 80 µl/ml. To this, culture media was added to each well to make a total volume of 300 µl mixture per well, gentle mixed and incubated in a 5% CO2-buffered and humidified incubator at 37^{o}C for 24, 48 and 72 hrs and cytotoxicity determined by MTT assay. For control wells, the same procedure was followed except that the cells were not exposed to extracts.

5.1.7 Methyl thiazol tetrazolium assay

The method of Meky *et al*. (2001) with modifications was employed in order to determine the cytotoxic potentials of the mycotoxins extracts and their combinations on the mononuclear cells. After 24, 48 and 72 hrs of incubation period of mononuclear cells, 30 µl MTT solution was added to each well and thoroughly mixed. The plates were again for 4 hrs at 37°C in 5% CO2-buffered and humidified incubator. After incubation, 50 µl dimethylsulphoxide (DMSO) was added to each well and mixed thoroughly in order to dissolve the formed blue crystals of formazan and incubated for 2 hrs in the same incubator. The optical absorbance density (OD) of wells was read using a Microplate Reader (Benchmark Plus Microplate Spectrophotometer) at

wavelengths 560 nm and 620 nm. The effect of mycotoxin exposure was calculated as % of cell stimulation of mycotoxin treated cells against control cells using the formula:

$$\% \text{ cell stimulation} = (ODM/ODN) \times 100$$

Where ODM is the OD value of mycotoxin-treated PHA stimulated cells and ODN is the OD of control cells (no mycotoxin) PHA-stimulated cells.

5.1.8 Statistical analysis

Results were analysed using one-way analysis of variance and the comparison of results between different groups of cells and to estimate the significance of the differences between the mean values of the various parameters as appropriate. Data was further graphically represented using SigmaPlot for Windows Version 10.0 (Systat Inc, 2006). Mean values among treatment groups were deemed to be different if the level of probability was <0.05.

5.2 RESULTS

To confirm the toxicity levels of mycotoxin producing extracts as reported in Chapter 4(Mycotoxigenic potentials of *Aspergillus*, *Fusarium* and *Penicillium* extracts isolated from houseflies and food commodities in Gauteng Province, South Africa), extracts were randomly selected based on the degree of mycotoxins produced on HPLC. Raw data for individual extracts in triplicates from houseflies, maize, porridge and water on human mononuclear cells are presented in Appendix III. In this case, the viability of untreated cells with *Aspergillus*, *Fusarium* and *Penicillium* extracts were recorded with 99.8%, 99.7% and 99.6% after 24, 48, and 72 hrs incubation period (Fig. 5.1, 5.2, 5.3, 5.4, 5.5, 5.6 and 5.7). It was observed that the viability of mononuclear cells after 72 hrs incubation was significantly reduced by *A. flavus* and *A. parasiticus* extracts from houseflies (42% and 47%), maize (27% and 39%), porridge (47% and 40%) and water (49% and 45%) as seen in Fig. (5.1, 5.2, 5.3 and 5.4). Whilst, exposure to *Aspergillus* extracts: *A. carbonarius*, *A. niger* and *A. ochraceus* revealed a decrease in cell viability reaching up to 56%, 61% and 49% in houseflies, 46%, 49% and 44% in maize, 65%, 67% and 69% in porridge and 53% in water isolates.

On the other hand, decreased in viability of mononuclear cells was observed when exposed to *Penicillium* extracts, particularly *P. verrucosum* from both houseflies, maize, porridge and water isolates at doses of 20, 40 and 80 µl over 24, 48 and 72 hrs (Fig. 5.5). Cell viability ranging between 75% and 86% in houseflies, 76% and 85% in maize, 74% and 82% in porridge and 76% and 84% in water isolates was observed over 48 hrs. Though decreased cell viability was observed at all concentrations, however, the highest decreased in cell viability was observed at the volume of 80 µl and exposure over 72 hrs ranging between 57% and 69%, 65% and 73%, 60% and 70%, 66% and 73%, respectively (Fig. 5.5).

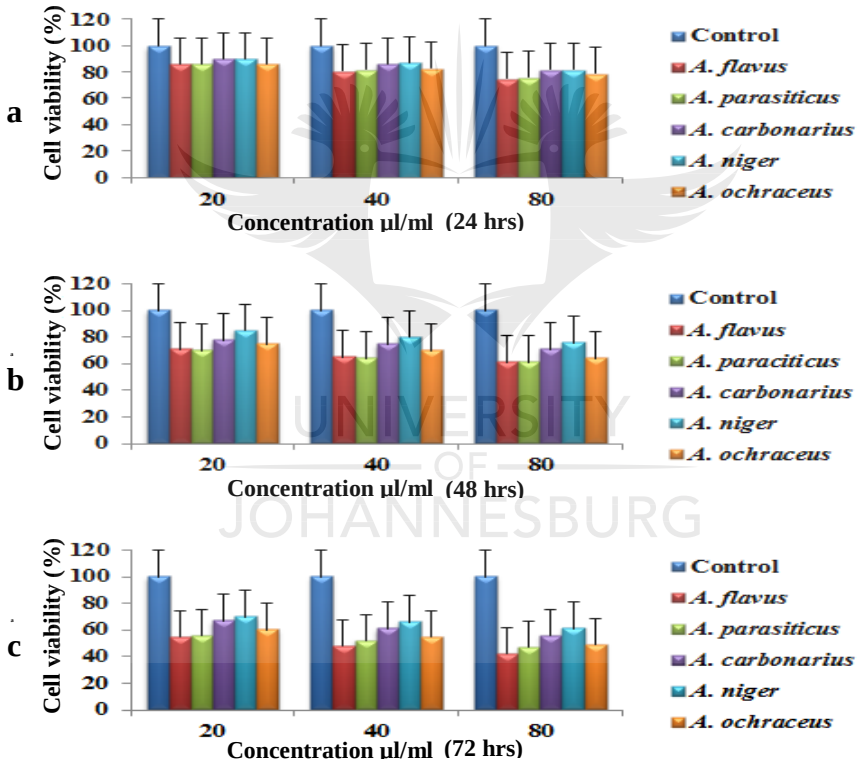


Figure 5.1 Effect of *Aspergillus* extracts from houseflies on human mononuclear cells.

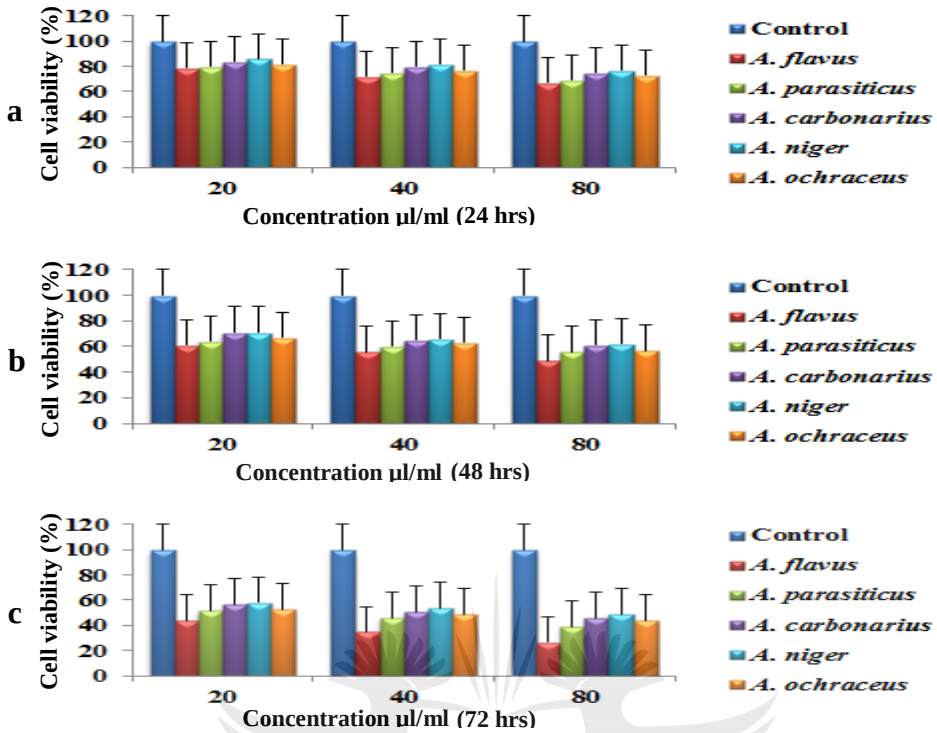


Figure 5.2 Effect of *Aspergillus* extracts from maize on human mononuclear cells.

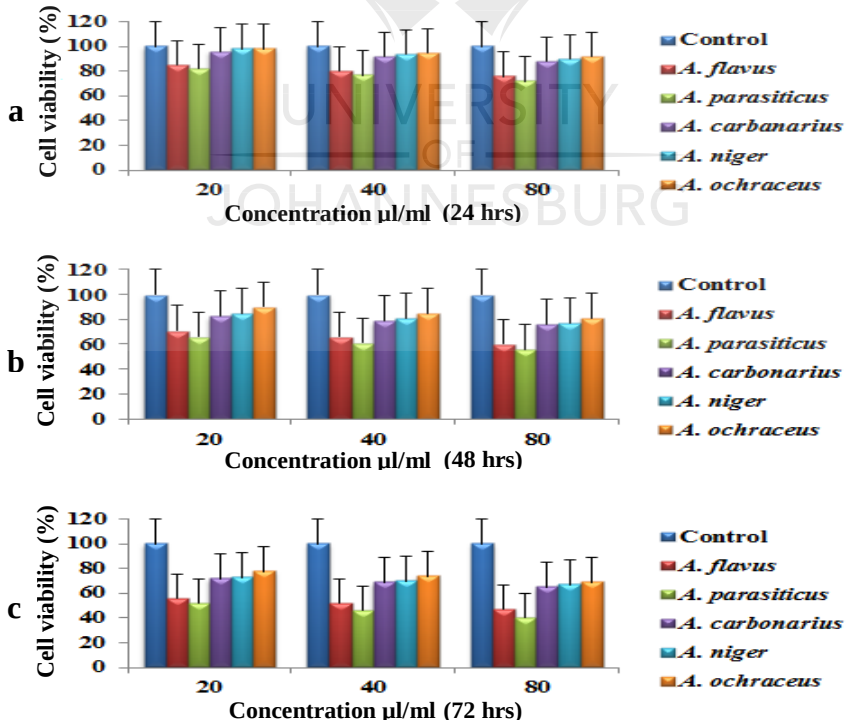


Figure 5.3 Effect of *Aspergillus* extracts from maize on human mononuclear cells.

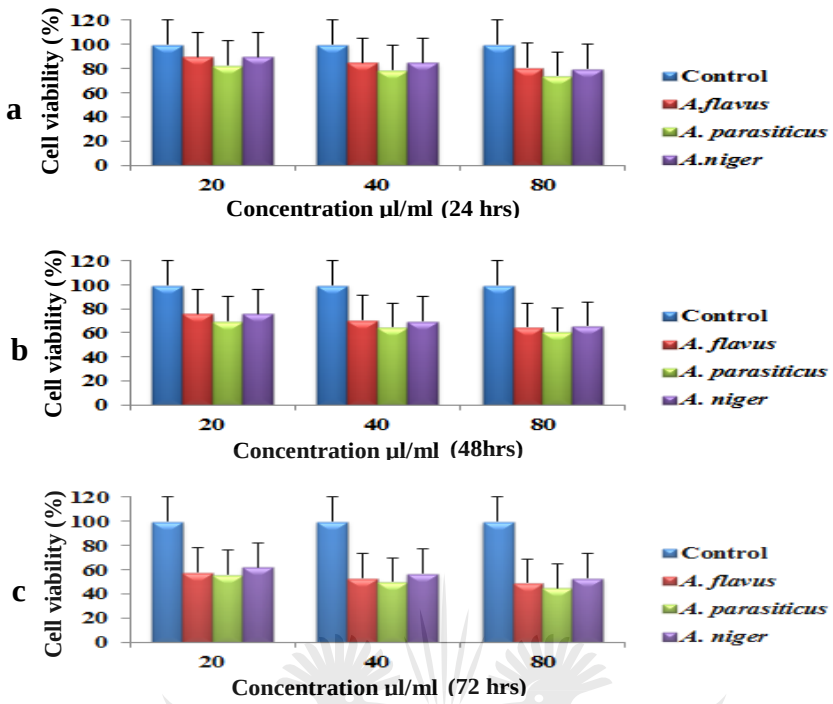


Figure 5.4 Effect of *Aspergillus* extracts from water on human mononuclear cells.

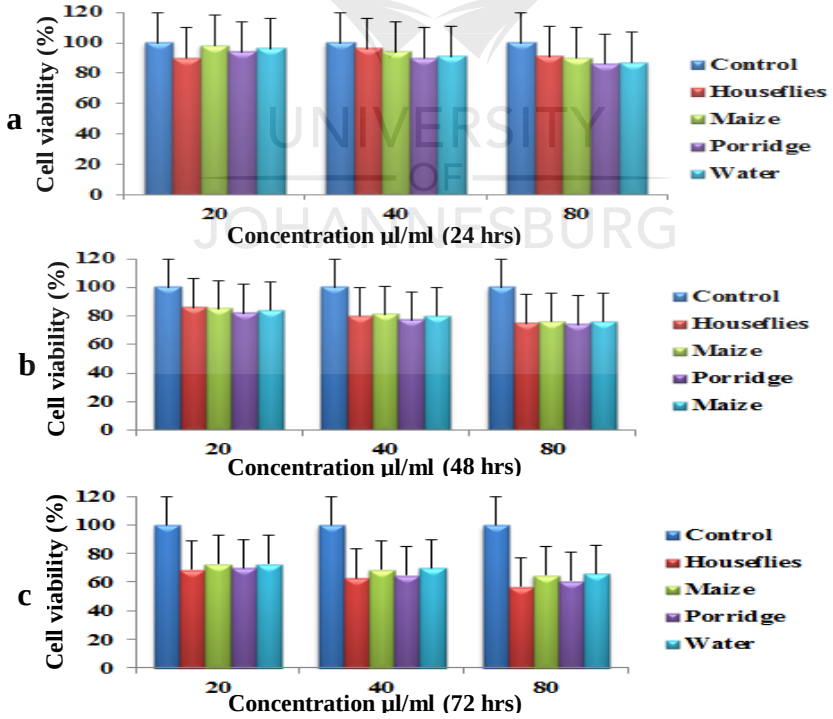


Figure 5.5 Effect of *Penicillium* extracts from houseflies, maize, porridge and water on human mononuclear cells.

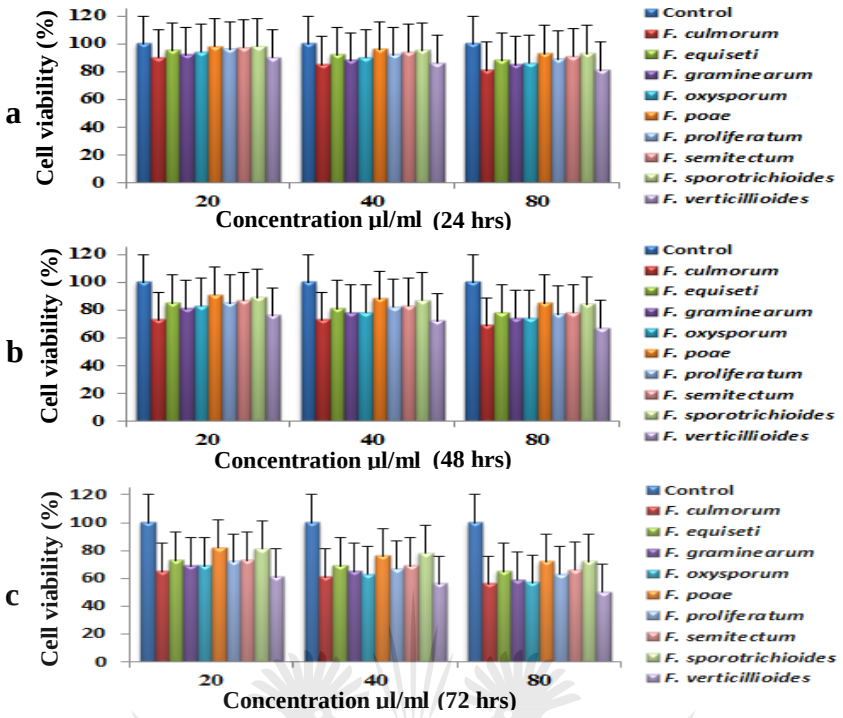


Figure 5.6 Effect of *Fusarium* extracts from houseflies on human mononuclear cells.

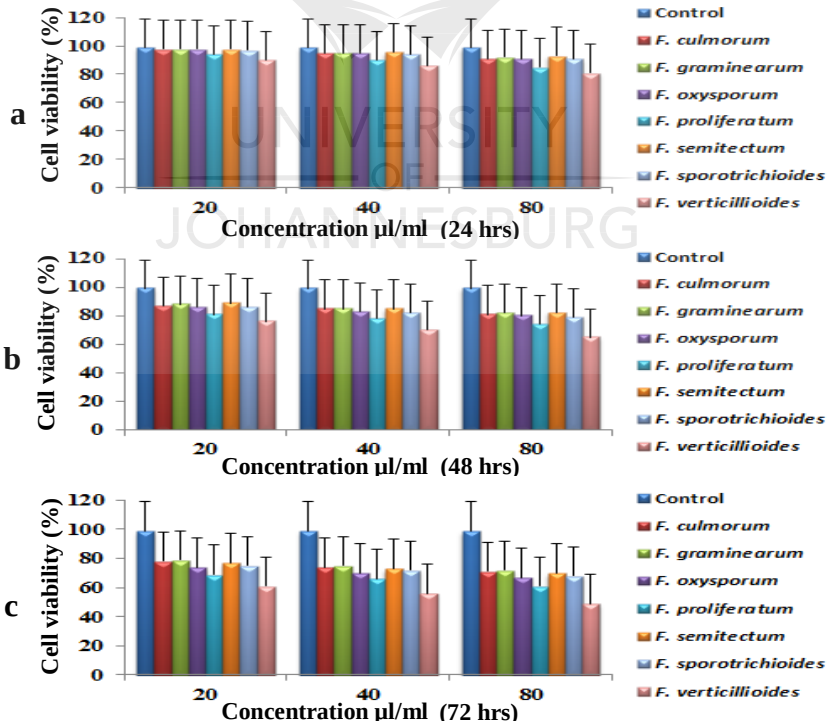


Figure 5.7 Effect of *Fusarium* extracts from maize on human mononuclear cells.

In the case of *Fusarium* extracts from houseflies, maize, porridge and water isolates , a great decrease in cell viability was recorded when exposed to at different doses over a period of 24, 48 and 72 hrs exposure (Fig. 5.6 and 5.7). As observed in Fig. 5.6, *F. verticillioides* and *F. proliferatum* extracts proved to be toxic at three different concentrations of 20, 40 and 80 µl over 24, 48 and 72 hrs incubation from houseflies isolates. Cell viability of human mononuclear cells was also reduced when exposed to *Fusarium* extracts from maize isolates over 72 hrs incubation reaching 49%, 61%, 67%, 68%, 70%, 71% and 72% for *F. verticillioides*, *F. proliferatum*, *F. oxysporum*, *F. sporotrichioides*, *F. semitectum*, *F. culmorum*, *F. graminearum* (Table 5.7). Although decreased cell viability was observed at all concentrations, cell viability decreased significantly ($p<0.05$) at the highest volume of 80 µl and over 72 hrs incubation when exposure to *Aspergillus*, *Fusarium* and *Penicillium* extracts. Furthermore, exposure to *Aspergillus*, *Fusarium* and *Penicillium* mycotoxins producing extracts from houseflies, maize, porridge and water isolates. at various concentrations, volumes and times induced a reduction of cell viability.

5.3 DISCUSSION

Mycotoxins are among the most important groups of natural toxins that can invariably cause toxic response when consumed by humans. The toxic effect of mycotoxins in the food chain is a food safety issue of a great concern worldwide. Aflatoxins, DON, FBs, OTA and ZEA are usually the monitored mycotoxins and stand out as the most common contaminants in a variety of foodstuffs. Thus far, studies have observed that these mycotoxins may cause acute toxicity or chronic effects which may pose significant threat on humans. In this study, cytotoxicity assay was conducted to assess the cytotoxic potential of *Aspergillus*, *Fusarium* and *Penicillium* extracts obtained from houseflies, maize, porridge and water isolates. According to the result obtained, exposure of mononuclear cells to *Fusarium* extracts containing DON, FB_1 and ZEA toxins from houseflies and maize isolates induced reduction in cell viability. However, it is worth mentioning that the fungus *F. verticillioides* produces several cytotoxic compounds other than the FB_1; such as ZEA. Furthermore, high concentrations of *F. verticillioides* extracts in houseflies and maize isolates (1064 µg/kg and 3920 µg/kg) were found to be more toxic compared to concentrations of *F. poae* and *F. sporotrichioides* (3 µg/kg and 8 µg/kg), *F. culmorum* and *F. oxysporum* (1275 µg/kg and 16 µg/kg), after 24 to 72 hrs of exposure. Similar

trend in decreased cell viability was observed by Lioi *et al*. (2004); Wright (2004) and Makun *et al*. (2011) that cell viability is influenced by concentration of the mycotoxin as well as the duration of the exposure of the mycotoxin. The decreased in cell viability induced by FB_1 on mononuclear cells might be elucidated by cytotoxic mechanism of FB_1 which is to mimic the structure of one of the primary sphingolipid bases, sphinganine, and thereby competes for the incorporation of sphinganine into ceramide and ultimately in complex sphingolipids (Riley *et al*., 2001). Due to this similarity, FB disrupts sphingolipid biosynthesis by inhibiting ceramide synthase (Wang *et al*., 1991; Mobio *et al*., 2000) thereby, causing the accumulation of these bases in the cell leading to cell deregulation, affecting important cell processes such as apoptotic cell differentiation and fatty acid metabolism (Merrill *et al*., 1996a). Other cellular processes such as phosphofructokinase action and arginosuccinate formation can also be inhibited by FB (Gopee *et al*., 2003; Dutton, 2009). On the other hand, the less toxic effect induced by ZEA on mononuclear cells in this study can be explained by the fact that ZEA is of a relatively low acute toxicity however, the health risk due to its oestrogenic effects. Hence, it competes with 17β-oestradiol for binding to the oestrogen receptor. As a result, binding of ZEA to the receptor may leads to a disordered protein synthesis of the oestrogen dependent proteins. Even though data on the combined effects of DON, FB_1 and ZEA from *Fusarium* extracts on human mononuclear cells is insufficient, reports of single and combined effects of DON, FB_1 and DON have been reported (Cetin and Bullerman, 2005; Kouadio *et al*., 2005; Bernabucci *et al*., 2011).

In the present study, *Aspergillus* and *Penicillium* extracts from houseflies, maize, porridge and water isolates containing high concentration of AFs and OTA reduced cell viability over time as exposure dose increased. Furthermore, AFs were found to be more toxic than OTA from both *Aspergillus* and *Penicillium* extracts. The reduction in cell viability when exposed to AFs and OTA producing extracts is in line with *in vitro* studies on human mononuclear cells and other cell lines conducted by various authors at different concentration over a period of hours (Scibelli *et al*., 2003; Lioi *et al*., 2004; Russo *et al*., 2005; Bouaziz *et al*., 2008; Cosimi *et al*., 2009). According to Groopmann and Kensler (1999) consumption of high levels of OTA and AFs producing extracts may lead to acute toxicity, whereas exposure to lower concentrations over a long period of time may have carcinogenic effects and to some extent leading to liver and kidney related diseases (Wagacha and Muthomi, 2008). In addition, studies on human populations

exposed to AFs producing extracts in their diet revealed a link between the high incidences of liver cancer in Africa with dietary ingestion of AFs (MERCK, 2006). Murphy *et al*. (2006) reported that AFs and OTA may induce immunosuppressive effects particularly in rural areas where Human Immuno Deficiency Virus/Acquired Immune Deficiency Syndrome (HIV/AIDS) is prevalent. Human lymphocytes play a vital role by being directly responsible for cellular support in the immune system. However, results revealed that the *Aspergillus* extracts decreased cell viability more as compared to those of *Fusarium* and *Penicillium* extracts. This could be accredited to the fact there is more than one mycotoxin contamination, such as aflatoxins, fumonisins and unknown ones, when not controlled these mycotoxins can be transmitted to humans through the ingestion of contaminated feeds and foods (Dashti *et al*., 2009).

The results in this study revealed that *Aspergillus*, *Fusarium* and *Penicillium* extracts in combination of other mycotoxins from houseflies, maize, porridge and water isolates were toxic to human mononuclear cells which would therefore affect human health. In addition, exposure of cells to mycotoxins induces cell damage and subsequently cell death depending on the toxicological properties of the mycotoxins. The decrease of cell viability observed on human mononuclear cells in this study might not only be related with the direct cytotoxicity effects of mycotoxins produced however, may be due to apoptosis of lymphocytes (Jones *et al*., 2001). The results in this study reveal that *Aspergillus*, *Fusarium* and *Penicillium* mycotoxins from houseflies, maize, porridge and water extracts were toxic to human mononuclear cells.

5.4 CONCLUSION

In general mycotoxins are cytotoxic to human mononuclear cells and might be a potential risk to human health. The findings obtained revealed that the *Aspergillus*, *Fusarium* and *Penicillium* fungal cultures obtained from houseflies, maize, porridge and water have cytotoxicity effects on PHA-stimulated human mononuclear cell viability using the MTT assay approach. The MTT assay also showed comparative results among different species, although it did not give more insights on the modes of biochemical actions of the tested mycotoxins. Through food humans are constantly exposed to combinations of mycotoxins in a variety of concentrations. Exposure to high mycotoxin concentrations often aggravates acute symptoms, which are occasionally recognised as mycotoxicoses. Chronic intake of sub-toxic mycotoxin concentrations might alter

various events at the cellular level, leading to biochemical and immunological impairments and possibly cancer. Then again, owing to the common nature of toxigenic fungi in the environment, mycotoxins are deliberated unavoidable contaminants in foods and feedstuffs. As a result, one of the most preventive measures to protect the public health is to revise current legislation levels of these mycotoxins on the basis of effective toxicological data.

CHAPTER SIX

Preliminary study on the use of bio-wipes to collect faecal samples for the detection of *Fusarium* toxin fumonisin B_1

ABSTRACT

Fusarium toxins with reference to fumonisin B_1 (FB_1) have long been regarded as contaminants of maize and maize-based related products intended for feedstuff and can cause intoxication, especially in humans. Therefore, effective quantitative methods for assessing dietary exposure of this toxic fungal metabolite are required. The objective of this investigation was to evaluate the effect of bio-wipe kit, which is a faecal material collection kit to detect the presence of FB_1. Faecal materials were collected from a rural farming community in Gauteng Province, South Africa. In total, 200 samples of faecal material were analysed for *Fusarium* species using a serial dilution method, while FB_1 was further analysed and quantified by reversed-phase thin layer chromatography (TLC) and high performance liquid chromatography (HPLC). The study revealed the presence of 11 different *Fusarium* species grown on potato dextrose agar culture medium of which, *F. verticillioides*, *F. Proliferatum*, producers of FB_1 and *F. oxysporum*, were the dominant species. Fumonisin B_1 was recorded at an incidence rate of 65% of the total using TLC. Results from HPLC revealed that 84% were positive at different ranges of concentration for FB_1. This review supports the use of bio-wipes as a rapid method of faecal collection to determine human exposure to FB_1.

Key words: Bio-wipes, *Fusarium*, Fumonisin B_1

6.0 INTRODUCTION

Fumonisin B_1 (FB_1) is the most commonly occurring metabolite of the family of mycotoxins called fumonisins (FBs) and is produced primarily by certain *Fusarium* species of mould including *F. verticillioides* and *F. proliferatum* (Bolger *et al*., 2001; Glenn, 2007). Despite the fact that FB_1 has been reported to be produced by other species, it is found as a natural contaminant mainly in maize and derived products intended for human food worldwide, particularly in South Africa (EHC, 2000; Kritzinger *et al*., 2003; Binder *et al*., 2007). The major *Fusarium* species (spp.) of economic importance is *F. verticillioides*, which is also a plant pathogen, causing a wide range of diseases on numerous host plants, such as vascular wilt, in addition to root and stem rot (Pascale *et al*., 2002; Schollenberger *et al*., 2006). *Fusarium* head blight (FHB), during pre and post-harvest of cereals, can be caused by several pathogens in the field and storage stage (Xu *et al*., 2005b). Moreover, fungal species of the genera *Fusarium* are often associated with the production of secondary metabolites that give adverse effects on humans and animals (Desjardins, 2006). Fumonisin B_1 contamination of maize has been reported worldwide at ppb and ppm levels. Therefore, human exposure from maize occurs at levels of µg to mg per day and is highest from home grown maize in South Africa and China (EC, 2000). Estimates of human exposure to FBs have been derived in some countries ranging from 0.017 to 0.089 parts per billion (ppb) body weight/day (bw/day) in Canada and a preliminary estimate of 0.08 ppb bw/day in USA. The mean daily intake is estimated to be 0.03 ppb bw/day in Switzerland. In South Africa the estimates ranged from 14 to 440 ppb bw/day, indicating that exposure to FB_1 is significantly higher than in the other countries in which human exposure assessments were performed (EHC, 2000).

Fumonisin B_1 is poorly absorbed in the gastro-intestinal tract (GIT) and is rapidly excreted mostly in its original form directly through the GIT via faeces, with some via the bile and small amounts in the urine (Shephard *et al*., 1994; EC, 2000; Stockmann-Juvalla and Savolainen, 2008). Also, FB_1 does not significantly permeate through the human skin and hence has no significant systemic health risk associated to dermal exposure, despite their widespread potential for skin contact and hazard toxicity (Boonen *et al*., 2012). It therefore, seems reasonable enough to look for FB_1 and its degradation products in faeces. Although effects of FB_1 on humans have not been demonstrated, toxicity studies of FB_1 have implicated consumption of fumonisin

contaminated commodity with elevated incidences of human oesophageal cancer, with an increasing incidence mainly in regions where maize is the base of the diet of populations (Sydenham *et al*., 1990; Rheeder *et al*., 1992; Marasas, 2001). However, such probable occurrence of FB_1 is correlated with the occurrence of a higher incidence of oesophageal cancer in regions of Transkei South Africa, China, and Northeast Italy (Chu and Li, 1994; Peraica *et al*., 1999a). Research studies conducted in Italy did not clearly establish correlation between the intake of FB_1 and the oesophageal cancer incidence (Pascale *et al*., 1995; Logrieco *et al*., 1995; EHC, 2000). In addition to cancer promoting consequences, consumption of fumonisin has been linked with neural tube defects (NTDs) in human babies (Zain, 2011; Wagacha and Muthomi, 2008) and cardiovascular problems (Fincham *et al*., 1992). According to Palanee, (2004) FB_1 has been revealed in the blood and tissues of rural patients with brain lesions. In a study conducted by Moodley *et al*. (2001), it was revealed that eclamptic patients have higher levels of FB_1 in serum than pre-eclamptic patients and normal pregnant women, suggesting that the presence of FB_1 in pregnant women could be responsible for eclampsia and possibly pre-eclampsia. A major concern could be that the rural populations in South Africa are exposed to mycotoxins, in particular FB_1 which can cause health effects. According to Dutton, (2009) the use of maize as a staple diet by black rural populations in South Africa is somehow linked to chronic disease considering the evidence that maize is contaminated with fungi producing FB_1, which is regarded as a maize disease in humans.

Based on studies carried out on faeces to determine FB1, conventional methods for sample collection have been used (Shephard *et al*., 1994b; Chelule *et al*., 2001; Mwanza, 2008). Chelule *et al*., (2001) measured FB_1 in staple maize and in faeces in rural and urban populations in Kwa-Zulu Natal, South Africa. From the analysis of faeces results it was concluded that such analysis may be a useful short-term biomarker for determining FB_1 exposure after 24 hours (hrs) of maize consumption, the toxin acting as its own marker. Considering the consequences of FB_1 ingestion, in order to prevent risk to humans, it is necessary to develop new strategies for quality control and safety assessment of the raw materials, and being able to detect toxic substances with minimum labour and costs. Fumonisin B_1 is a polar molecule and is usually extracted with mixtures of polar solvents, such as acetonitrile and water in different combinations and proportions, according to Scudamore *et al*. (1997) and Cortez-Rocha *et al*. (2003). Besides,

several authors have experienced difficulties with respect to the extraction, purification and detection of FB_1. As a result, rapid and sensitive analytical procedures such as reverse-phase thin layer chromatography (Shephard and Sewram, 2004) and high performance liquid chromatography (Shephard *et al*., 1994) are currently being used to confirm and quantify FB_1. Furthermore, health concerns resulting from the finding of FB_1 and the observation of both acute and chronic effects particularly in humans has impelled the research effort focusing on method development. Fumonisin B_1 analysis is important to reduce the consumption of contaminated feedstuffs. However, method development and evaluation for FB_1 is not a simple task. The objective of this investigation was to evaluate the effect on the use of bio-wipe kit, which is a faecal material collection kit to detect the presence of FB_1. Since FB_1 when absorbed is readily excreted into the faeces it therefore, justifies the need to quantify FB_1 in the bio-wipes.

6.2 MATERIALS AND METHODS

6.2.1 Chemicals and reagents

All chemicals and reagents were at least of HPLC grade, unless otherwise specified.

(a) ***Bio-wipe kit*:** Bio-wipes consist of a 10 x 10 cm square barrier side and an absorbent side with a bright orange, non-slip plastic backing (Thermo Fisher Scientific, USA).

(b) ***Removal of bio-wipe materials*:** Phosphate buffered saline (PBS) (pH 7.4), Tween buffer and safe-lock Eppendorf tubes obtained from (Sigma-Aldrich, Louis, MO, USA; Associated Chemical Enterprises (Pty) Ltd., Johannesburg, Gauteng, South Africa and Eppendorf AG, Hamburg, Germany).

(c) ***Fumonisin B_1 screening equipment and materials*:** Bench shaker (LABCON GmbH, Heppenheim, Germany), Filter paper (Whatman no. 4, Sigma-Aldrich), separating funnels and flasks (Lasec, SA; pH meter, Thermo Fisher Scientific, USA; Eppendorf tips, Eppendorf AG and amber vials, National Scientific, USA).

(d) ***Thin Layer Chromatography mobile phase and plates*:** methanol/4% aqueous potassium chloride (70:30, v/v), reversed-phase (C18) 20cm by 20cm TLC plates, (Whatmann

LKC18 with pre-concentration zone, (Merck, Germany), chromatography tanks (Camag Ltd), adjustable hot air drier and UV light box.

(e) ***Derivatizing agent for Fumonisin B_1 on thin layer chromatography (TLC)*:** Fluorescamine prepared by dissolving 0.04 g in 1 ml of ACN (Sigma-Aldrich).

(f) ***Solvents used for extraction and high performance liquid chromatography (HPLC) purposes*:** Acetonitrile (ACN) and Dichloro-methane (DCM) and Methanol (MeOH) obtained from Sigma-Aldrich (Louis, MO, USA); Sulphuric acid (H_2SO_4) and Acetic acid (A.A) obtained from (Merck, Germany).

(g) ***High performance liquid chromatography purified water*:** HPLC-grade H_2O was obtained from water passed through a MilliQ water purification system (Millipore Ltd., Bedford, MA, USA).

(h) ***Mobile phase for high performance liquid chromatography*:** CH_3OH/sodium di-hydrogen phosphate (Na_2HPO_4), (80/20, v/v), prepared by dissolving 2.84 g of Na_2HPO_4 in 200 ml of HPLC-grade H_2O and 800 ml methanol to make up a total volume of 1000 ml (pH adjusted to 3.4 using 1 M H_2SO_4).

(i) ***Mycotoxin standard*:** Fumonisin B_1 standard was obtained from PROMEC (Programme on Mycotoxins and Experimental Carcinogenesis, MRC, South Africa).

(j) ***Derivatising agent for Fumonisin B_1 on high performance liquid chromatography*:** o-phthaldialdehyde (OPA) reagent prepared by dissolving 0.04 g of OPA in 1 ml CH_3OH and diluted with 0.1 M sodium tetraborate ($Na_2B_2O_4$) prepared by dissolving 0.19 g of $Na_2B_2O_4$ in 5 ml of HPLC-grade H_2O and 50 µl mercaptoethanol (Merck, Germany).

(k) ***High performance liquid chromatography equipment*:** The HPLC equipment used was purchased from Shimadzu Corporation (Kyoto, Japan) LC-20AB liquid chromatograph equipped with CBM-20A communication bus module, LC-20AB degasser, CTO-20A column oven, Nova-Pak 4mm C18 reversed phase separation column (250 × 4.6 mm, with particle size of the sorbent 5µm), SIL-20A auto sampler, RF-10AxL fluorescence

detector linked to LC solutions version 1.22 Software Release (Excitation and Emission wavelengths were 335 and 440 nm; flow rate of 1 ml/minute (min).

6.2.2 Sampling and study area

To accomplish the objective of the study, a total of 200 bio-wipe samples were collected from a study area in the Gauteng province, South Africa which consisted of 84 houses. Bio-wipe kits were distributed to participants to use as toilet papers to recover the faecal matter as demonstrated in Fig. 6.1. Samples were collected during peak and off-peak hours to ensure that all participants are sampled. All participants were mostly females, who retained the right whether to withdraw or to continue partake in the study and personal information was coded and confidential. The used bio-wipes were placed in Ziploc plastic bags, placed in cooler boxes and transported to the Food, Environment and Health Research (FEHR) laboratory, University of Johannesburg, South Africa, where they were preserved at -20^{o}C until analysed. The study was approved by the Faculty of Health Sciences Ethical Committee, University of Johannesburg.

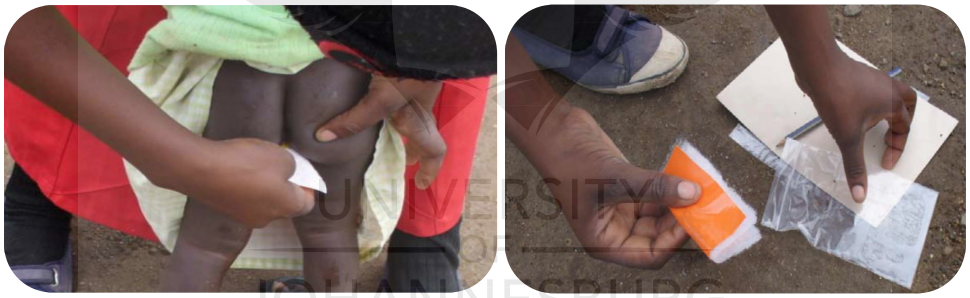


Figure 6.1 Demonstration on the usage and collection of bio-wipes, photographed by (SIK, Meita).

6.2.3 Extraction of faecal material from bio-wipes

In order to extract the faecal material, bio-wipes were placed in 150 mm sterile Petri dish with the faecal material side facing upwards. Six ml of 0.1 % of Tween buffer + phosphate buffer saline (Sigma-Aldrich, Louis, MO, USA) were pipetted onto the absorbent side of the bio-wipe. The faecal material was suspended from the bio-wipe by pipetting 3 ml volumes of the buffer from the Petri dish onto the bio-wipe. The procedure was repeated until most of the faecal matter was removed from the surface of the bio-wipe. The bio-wipes were then squeezed to remove the suspended faecal material and the entire excess buffer that may have been absorbed into the bio-

wipes. An aliquot of the buffer containing the faecal material was pipetted into 3 ml safe-lock Eppendorf tubes (Eppendorf AG, Hamburg, Germany). Two equal volumes (1 ml) of the aliquot was transferred into 2 separate sterile freeze-drying vials, freeze-dried for overnight for FB_1 extraction and fungal isolation. The weight of the vial containing the bio-wipe sample was measured before (mean= 12.41 g) and after freeze-drying (mean= 11.40 g) to determine the final faecal material mass of the bio-wipe (1.01 g). The remaining 1 ml was preserved at -20^{o}C for future use.

6.3 FUNGAL SCREENING

6.3.1 Culture media

Two media were used, Potato Dextrose Agar (PDA) as general culture medium prepared according to the manufacturer's instructions and Ohio Agricultural and Experimental Station agar (OAESA) prepared according to Kaufman *et al*., 1963. Both media were prepared under asceptic conditions.

6.3.2 Isolation of *Fusarium* fungi

Under sterilized conditions, the method described by Burgess *et al*. (1994) was followed with minor modification for plating procedures, media and incubation conditions. One gram of the powdered faecal material sample was diluted in 9ml of sterile Ringer's solution, vortexed and serially diluted further to 10^{-6}. One ml suspension from each of the test tubes was pipetted on OAESA and PDA and incubated for seven (7) days under standard growth conditions for the isolation and growth of *Fusarium* spp. After 7 days of incubation, the fungal colonies on the plates were counted using a colony counter to determine the colony forming unit per gram of sample colony forming units per gram (CFU/g). Sporulated and isolated fungal colonies were further sub-cultured and incubated on PDA at 25^{o}C for 7 days to initiate pure cultures for identification of *Fusarium* species. The fungal load was calculated and expressed in colony forming units per gram of sample (CFU/g) as:

$$\text{CFU/g} = \frac{\text{Number of colonies x reciprocal of the dilution factor}}{\text{Plating volume (1ml)}}$$

Sporulated and isolated fungal colonies were further sub-cultured and incubated on PDA at 25^{o}C for 7 days to initiate pure cultures for identification of *Fusarium* spp.

6.3.3 Conventional species identification

The microscopic and macroscopic characteristics of *Fusarium* species such as micro and macro conidia, as well as the presence of phialides, types of conidiophores, colony colour and texture of each isolate were examined under a light microscope following the keys of Leslie and Summerell (2006) and Diba *et al*. (2007). The germinated single fungal isolates were stained with lactophenol blue mounting between the slides and the slide covers for identification.

6.3.4 Fumonisin B_1 extraction procedure

One gram of the freeze-dried faecal material samples were each mixed with 2 ml of acetonitrile/water (1/1, v/v) and shaken on a bench shaker for 1 hr. After shaking the combined samples were acidified to pH 2.9-3.2 using 1 M of sulphuric acid. After adjusting the pH, acidified samples were poured into 50 ml separating flasks and extracted twice with dichloro-methane, shaken and allowed to stand. The bottom layer containing the extracts was collected and 2 ml of acetonitrile (ACN) was added. The combined extracts with ACN were then filtered through a Whatman no. 4 filter paper (125 mm diameter, Sigma-Aldrich). The filtered extracts were transferred into capped amber glass vials and dried at 60^{o}C by passing through nitrogen (N_2) gas. The extracts were then stored at 4^{o}C for further analysis.

Figure 6.2 Side view (*left*) and front view (*right*) illustration of extraction of bio-wipes on a bench shaker.

6.4 FUMONISIN B_1 ANALYSES

6.4.1 Identification of fumonisin B_1 in faecal materials by reversed-phase TLC

Thin layer chromatography (TLC) developing tanks were prepared in accordance with the method described by Shephard and Sewram, (2004), by placing a Whatman no. 4 filter paper (24 cm diameter) in the tank, adding methanol/4 % aqueous potassium chloride (70:30, v/v) mobile phase and allowing at least 4 hr for equilibration. The FB_1 extracts were re-dissolved in 200 µl of methanol and vortexed for 1 min. Twenty-five (25) µl of extracts or FB_1 standard solution were derivatized with 0.1 M borate buffer (25 µl) and fluorescamine solution (25 µl) and allowed to stand for 1 min at room temperature. Thereafter, 15 µl of the mixture was spotted within the pre-concentrated zone on the reversed-phase TLC plates (Whatmann LKC_{18}, Merck, Germany) and inserted into the equilibrated developing tanks. The plates were then allowed to develop to at least 10 cm above the pre-concentrated zone to ensure complete saturation. The plates were run on one-dimensional mode, dried under a stream of warm air and examined under long wavelength ultraviolet light. The fluorescence spot of FB_1 was recorded and the retardation factor (R_F) of the individual spot was calculated as the proportion of the distance travelled by the analyte to that of the developing solvent, which was compared with that obtained for FB_1 standard solution. Thereafter, extracts were then dried using N_2 gas as previously described.

6.4.2 High performance liquid chromatography analysis of FB_1

Quantification of FB_1 present in faecal material extracts was further performed by HPLC, using a modification of a previously described procedure of Visconti *et al*. (2001). Briefly, extracts were re-dissolved in 1 ml HPLC grade methanol and 250 µl aliquot of each of the faecal material extracts or standard (10 µg/ml) were pipetted separately into an HPLC vials containing 50 µl derivatizing solution OPA added and vortexed for 30 s. The reaction mixtures were injected onto the HPLC separation column within 2 min of adding OPA, due to its instability. The identification and quantification of FB_1 was observed by comparison of the constant retention times and peak areas in the bio-wipe samples with those observed for FB_1 standard and using the relevant calibration curve.

6.4.3 Determination of recoveries of FB_1 on spiked samples

To determine the effectiveness of the methods employed for extraction of FB_1 from faecal material samples, 100 µg/ml of FB_1 standard was spiked in triplicate thoroughly mixed on blank faecal material samples and FB_1 was extracted following the method previously described above.

6.4.4 Data analysis

Mean values of fungal load were calculated by adding the total number of CFU which was the divided by the number of plates from each sample. A one-way analysis of variance (ANOVA) was performed to derive mean values of FB_1 levels for positive samples (Systat Software Inc. 2006). Mean values among sample types were deemed to have significant differences if $p \geq 0.05$.

6.5 RESULTS

6.5.1 Incidences of *Fusarium* species in faecal materials

A total of 200 bio-wipe samples analysed for fungal isolation and identification, data displayed the presence of 11 different *Fusarium* spp. Figure 6.3 confirms the characteristics of some isolated *Fusarium* strains grown on PDA culture medium and microscopic features isolated. Among the *Fusarium* spp. isolated, *F. verticillioides*, *F. proliferatum* and *F. oxysporum* were the most dominant strains.

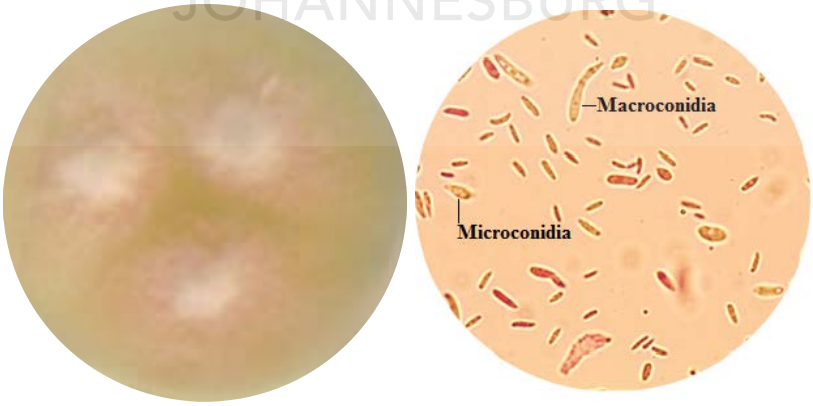


Figure 6.3 Macroscopic and microscopic characteristics of *Fusarium oxysporum* colony feature on PDA medium, after 7 days of incubation at 25^{0}C (*left*); Macroconidia and micro conidia (*right*).

Results in this study displayed the presence of 11 different *Fusarium* species. Among the *Fusarium* species isolated, *F. verticillioides*, *F. proliferatum* and *F. oxysporum* were the most dominant strains. *Fusarium* contamination was evaluated based on the percentage incidences and contamination levels. Furthermore, a majority of the *Fusarium* species were identified as *F. verticillioides*, *F. proliferatum*, *F. oxysporum*, *F. graminearum*, *F. dimerum* and *F. solani*. However, *F. equiseti*, *F. poae*, *F. sporotrichioides*, *F. semitectum* and *F. nivale* were also identified. Although *F. sporotrichioides* was among the less dominant isolated species; heavy contamination in those infected samples of this fungus was observed followed by *F. verticillioides*, *F. semitectum*, *F. nivale* and *F. graminearum*. For other isolated species, lower mean contamination levels were detected for *F. equiseti*, *F. oxysporum*, *F. solani*, *F. poae* and *F. proliferatum* (Table 6.1).

Table 6.1 *Fusarium* species isolated in faecal material from Gauteng Province, South Africa

Incidence rate and levels of contamination			
Dominant *Fusarium* species	**Number of species**	**Percentage (%)**	**Mean fungal load (CFU/g)[a]**
F. dimerum	16	8	4.0×10^5
F. equiseti	9	5	3.8×10^5
F. graminearum	20	10	4.3×10^5
F. nivale	5	3	4.5×10^5
F. oxysporum	22	11	3.6×10^5
F. poae	9	5	2.8×10^5
F. proliferatum	32	16	1.2×10^6
F. solani	16	8	3.4×10^5
F. semitectum	8	4	5.0×10^5
F. sporotrichioides	9	5	8.2×10^5
F. verticillioides	50	25	5.4×10^5
Total	**200**	**100**	5.7×10^6

Notes: [a]CFU/g: colony forming unit per g of sample; n: number of samples analysed. Results are expressed as percentage of isolates relative to the total number of isolates.

6.5.1 Occurrence of FB_1 in faecal materials.

6.5.2 Thin layer chromatography

Thin Layer Chromatography and HPLC techniques were conducted to determine and quantify the presence of FB_1 in bio-wipe samples. Screening of FB_1 in extracts on the Reversed-phase TLC plate derivatised with fluorescamine was visualised under long-wave ultra violet light. Data presented in the study revealed that a number of samples were positive with incidence rates of 65% (n=130). These incidence rates were further confirmed by HPLC analysis. Fumonisin B_1 appeared as bright yellowish-green fluorescent bands as demonstrated in Fig. 6.4. Moreover, most of the TLC plates visualised indicated the presence of FB_1 at trace levels in some samples. Fumonisin B_1 was also visualised by comparing the fluorescence intensity of spots and R_F for samples (A-B) with that of FB_1 standard (C) on the same plate. The R_F value of FB_1 standard measured under ultra violet light was 0.39, while those of all the positive samples had R_F values ranging from 0.37-0.45.

Figure 6.4 Reversed-phase one dimensional thin layer chromatography plate showing fumonisin B_1 band (circle): (A-B) contaminated faecal material samples and (C) fumonisin B_1 standard.

6.5.3 High performance liquid chromatography

The quantities and concentrations detected on HPLC were based on the chromatogram of calibration curve. The linear relationship between peak area and concentration of FB_1 was represented by the equation Y= aX + b, a= 7.032772e-006, b= 0.0, with a correlation coefficient (R^2) of 0.99999918, chromatogram presented in Fig. 6.5.

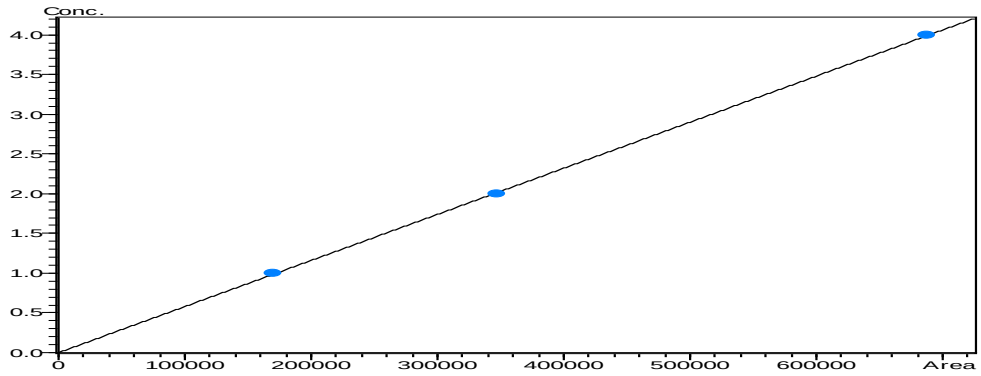


Figure 6.5 Calibration curve of fumonisin B_1 standard for the determination of fumonisin B_1 by high performance liquid chromatography (correlation coefficient: R^2 = 0.9999918).

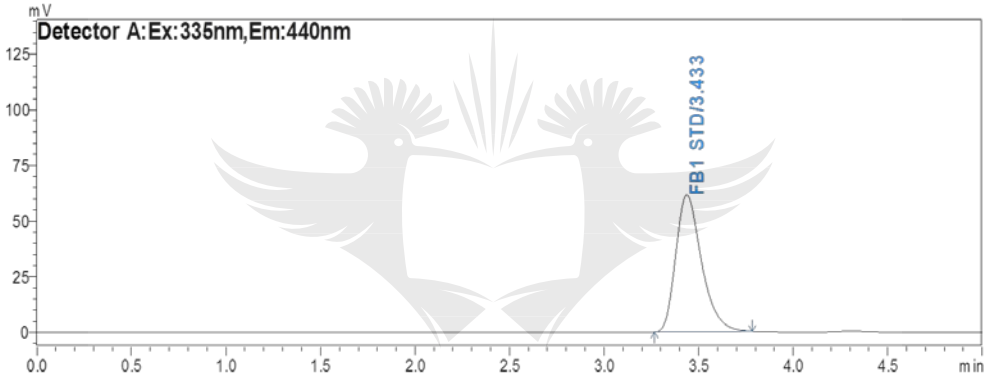


Figure 6.6 Chromatogram of fumonisin B_1 standard (1 µl of extract injected) at retention time of 3.4 min.

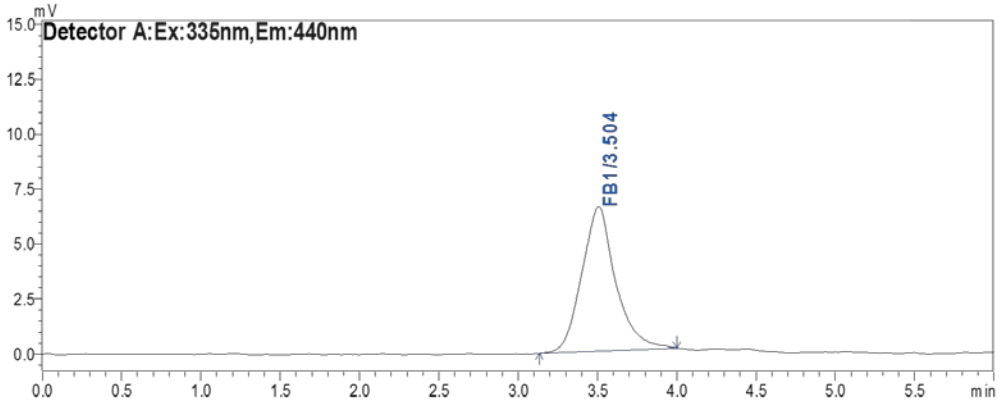


Figure 6.7 Chromatogram of fumonisin B_1 in a faecal material sample (1 µl of extract injected) at retention time of 3.5 min.

High performance liquid chromatography analysis showed a peak of FB_1 standard at a retention time of 3.4 min (Fig. 6.6). A similar peak for FB_1 in the faecal material sample was observed at 3.4 min (Fig. 6.7). The limits of detection (LOD) of FB_1, defined as the lowest concentration that the analytical process can reliably differentiate from background levels, was determined for those concentrations that provided a signal-to-noise ratio of 3:1. Recovery for FB_1 was determined by the extraction from a blank sample spiked with known levels of FB_1 standards to confirm the stability of the results. The mean percentage recovered was found to be 90.3±2.1% for the faecal material samples. Samples that were negative by TLC were positive when analysed by HPLC. This could be accredited to the fact that HPLC is more sensitive than TLC.

Data on HPLC revealed that of the 200 faecal material samples analysed, 168 (84%) were positive at different concentrations, while the remaining 32 samples were negative for FB_1. The pattern of FB_1 levels detected in faecal material samples per household ranged from 20 to 4133 µg/kg as presented in Table 6.2. Data revealed that FB_1 contents from faecal material samples were found to be high with contamination levels of 4133 µg/kg with the highest mean value of 1740 µg/kg. Furthermore, higher proportion of FB_1 when compared to other households may be deduced to different handling or preparation methods practiced individually by households prior to consumption.

Table 6.2 High performance liquid chromatography detection of fumonisin B_1 per household in faecal material samples from Gauteng Province, South Africa

		Fumonisin B_1	
HH Site[#]	**Sample no.***	**Range of faecal material (µg/kg)**	**Mean of faecal material (µg/kg)**
B2	5 (4)	789 to 1030	907
B4	1 (0)	n.d	n.d
B6	3 (1)	660	660
B10	1 (0)	n.d	n.d
B11	3 (2)	310 to 587	449
B12	1 (1)	20	20
B14	3 (2)	27 to 543	285
DB2	2 (2)	520 to 760	640
DB3	3 (3)	37 to 530	284
DB4	1 (1)	37	37
DB6	3 (2)	467 to 1667	1067
DB7	2 (1)	1067	1067
DB8	3 (3)	560 to 873	707
DB9	1 (1)	913	913
DB11	1 (1)	27	27
DB12	2 (1)	723	723
DB13	1 (0)	n.d	n.d
J1	5 (5)	567 to 957	792
J2	8 (8)	207 to 887	560
J3	5 (5)	757 to 1403	1015
J4	1 (1)	487	487
J6	5 (5)	550 to 2657	1295
J9	5 (5)	490 to 4133	1365
J11	4 (4)	233 to 1150	643
J12A	6 (5)	310 to 1057	635

[#]HH: House Hold; * Number = number of samples taken; Values in bracket represent the number of positive samples; no.: number; n.d: not detected; Mean values are for positive samples.

Table 6.2 Continued……..

Fumonisin B_1			
HH Site[#]	**Sample no.***	**Range of faecal material (µg/kg)**	**Mean of faecal material (µg/kg)**
J12B	4 (4)	563 to 2290	1132
J13	1 (1)	23	23
J14	2 (2)	55 to 67	61
J15	2 (2)	723 to 940	832
J16	3 (3)	430 to 1167	725
J17	3 (2)	1357 to 1513	1435
J19	1 (1)	210	210
J20	1 (1)	430	430
J21	4 (4)	520 to 973	738
J22	2 (2)	777 to 810	794
J23	2 (2)	340 to 430	385
J24	3 (3)	603 to 1890	1199
LB1	6 (6)	287 to 3900	1552
LB2	4 (3)	370 to 923	510
LB3	2 (2)	213 to 326	270
LB4	1 (1)	23	23
LB5	7 (6)	163 to 617	316
LB6	4 (2)	593 to 2887	1740
LB8	5 (5)	20 to 703	312
LB9	6 (4)	270 to 1347	645
LB10	4 (3)	37 to 873	456
LB12	3 (2)	3 to 33	18
LB13	6 (4)	273 to 4107	1447
ML8	4 (3)	23 to 83	54
ML9	1 (0)	n.d	n.d
ML10	1 (1)	68	68

[#]HH: House Hold; * Number = number of samples taken; Values in bracket represent the number of positive samples; no.: number; n.d: not detected; Mean values are for positive samples.

Table 6.2 Continued……..

		Fumonisin B_1	
HH Site[#]	**Sample no.***	**Range of faecal material (µg/kg)**	**Mean of faecal material (µg/kg)**
ML13	1 (1)	557	557
ML17	2 (1)	567	567
ML18	4 (3)	27 to 1003	532
ML19	1 (1)	30	30
ML20	1 (1)	20	20
ML21	6 (5)	30 to 460	210
ML23	3 (2)	203 to 997	600
P2	3 (2)	270 to 507	389
P4	1 (1)	50	50
P5	1 (1)	670	670
P6	2 (2)	413 to 809	611
P7	7 (7)	20 to 1154	389
WB2	3 (2)	577 to 930	754
WB3	2 (2)	246 to 272	278
WB4	2 (2)	24 to 1107	566
WB8	1 (1)	523	523
WB9	2 (2)	230 to 1090	660
WB10	2 (2)	30 to 55	43

[#]HH: House Hold; * Number = number of samples taken; Values in bracket represent the number of positive samples; no.: number; n.d: not detected; Mean values are for positive samples.

6.6 DISCUSSION

Fusarium species are well-known by researchers whose main interest focus on fungi associated with plants and soil, due to their characteristics such as the phialides, multi-septate and slightly curved macro and micro conidia (Moss, 2002). The incidences and the fungal load of *Fusarium* species isolated from faecal material samples are reported in Table 6.1. In the present study *Fusarium* species isolated and identified have been considered some of the most important

pathogenic fungal species that affect staple food commodities. Noteworthy, from various studies conducted in South Africa (Dutton *et al*., 2001; Marasas, 2001) and in some parts of the world (Desjardins, 2006), these species have been also reported as major contaminants in maize, especially those belonging to the *F. verticillioides* at high contamination levels. Maize is the most agricultural product grown in rural communities of South Africa and serves as the main staple diet. Furthermore, it was also recognised that the maize grown in the study area in Gauteng, South Africa is susceptible to contamination by various fungi, particularly *Fusarium* species. *Fusarium verticillioides* and *F. proliferatum* were the most often isolated *Fusarium* species with *F. oxysporum* being the third (Table 6.1). The prevalence of *Fusarium* species in the faecal material samples could be due to exposure of different spores that resist the conditions in the digestive tract, indicating that the conditions in the digestive tract promote germination and sporulation upon exiting the stomach (Tam *et al*., 2006). Another reason could be the fact that *Fusarium*-contaminated intake by the same population could be attained from other sources, for example roasted corn and home brewed maize-based beer. Studies in mice have shown that animals given a fixed oral dose of spores excreted more spores in their faeces than were administered (Hoa *et al*., 2001). In this case, a very important source of exposure to FB_1 in humans comes after consumption of contaminated foodstuffs and through inhaled fungal spores (Reddy *et al*., 2012). Additionally, there is insufficient data to understand fungal absorption, effects and possible interactions between different fungi spores in the digestive tract and on fungal colonization of human excreta, thus making it possible to speculate. Rafferty *et al*. (1994) conducted a study which confirmed the presence of fungal spores in animal faeces. This may be elucidated by the gastric functioning, which is polygastric for animals such as sheep and cattle. *Fusarium verticillioides* and *F. proliferatum* identified in the study are the most important producers of FB_1 because of their occurrences in maize (D'Mello, 2003). A summary of global incidence of *Fusarium* mycotoxins in foods intended for human consumption, in addition to other agricultural foodstuffs has been reviewed by Soriano and Dragacci (2004). What should be of great concern is that, most of the analysed faecal material samples had co-existence of more than one *Fusarium* species and hence suggests possibly co-contamination of more than one *Fusarium* toxin other than FB_1.

Results on one dimensional thin layer chromatography confirming the presence of FB_1 are demonstrated in Figure 6.4. Results revealed that many samples (102 of 200) were positive with incidence rates of 51% for FB_1. Shephard and Sewram (2004) developed a method using reversed-phase thin layer chromatography for determination of FB_1 in maize by means of strong anion exchange solid-phase extraction and clean-up. Ever since their report, this method has improved the determination of FB_1 in faeces (Phoku *et al*., 2012). Furthermore, in this study it was found that FB_1 could also be rapidly extracted, detected and quantified by the use of reverse-phased thin layer chromatography and high performance liquid chromatography without any clean-up, which has an advantage of serving time and financial implications. The quantification of FB_1 revealed higher contaminated levels in all samples analysed by HPLC (Table 6.2), with the results ranging from 20 to 4133 µg/kg. The highest and lowest ranges obtained in these results may be attributed to the quantity of consumed food contaminated with FB_1 and in addition to the number of samples analysed. Besides, these results are presented presumably according to how much FB_1 is excreted via faeces by an individual per day, suggesting that a portion of the FB_1 is absorbed in the gastro-intestinal tract. In addition, higher proportion of this mycotoxin when compared to other households may be attributed to different handling or preparation methods practiced individually by households prior to consumption. The presence of FB_1 in faecal material samples concurs with the results obtained in a study conducted by Phoku *et al*. (2012) in which fumonisins mycotoxins were detected in human faeces from Mapate village in Limpopo Province, South Africa. Also, may be compared to those obtained from previous work conducted by Chelule *et al*. (2001) whereby 35% of the samples had incidence levels ranging from 6000 to 20000 µg/kg. However, this has been observed in the study where HPLC was used to determine levels of FB_1 in samples with level of concentration ranging from 0.3-464 µg/kg.

Moreover, from the table above, it is very difficult to determine the proportion of FB_1 excreted in this study, because individuals from the different households did not consume food contaminated with known concentrations of FB_1. This study continues to reveal that a much higher proportion of FB_1 consumed by the population in the study area appears to be excreted via faeces. According to several studies, FB_1 is poorly absorbed from the diet and when absorbed it is quickly excreted into the faeces, in the bile, while some remains in the kidney, liver (Shephard *et*

al., 1994) and also in the blood (Moodley *et al*., 2001; Palanee, 2004). From the results, it is clearly indicated that the people from Gauteng Province are not only consuming foodstuffs that are contaminated with high levels of FB_1 but be exposed to high total levels, over a period of time, which is due to continuous exposure to FB_1 in their diet. Higher incidence levels of FB_1 in specific products such as maize and maize-based products from rural populations of South Africa have been reported by several authors (Marasas, 2001; Shephard *et al*., 2004; Phoku *et al*, 2012). D'Mello *et al*. (2003) reviewed high levels between 50-46900 µg/kg in maize from the Eastern Cape Province, while Phoku *et al*. (2012) reported significantly high levels of 101-53,863 µg/kg and 0.2-20 µg/kg in maize and porridge respectively from Limpopo Province of South Africa. Furthermore, what of concern is that the presence of fungal contamination, especially *Fusarium* species is a potential risk for human health, because they produce FB_1, a toxic and carcinogenic metabolite (IARC, 2002; Jackson *et al*., 2000). The carcinogenic effects of FB_1 on the oesophagus and developments of Idiopathic congestive Cardiopathy (ICC) had been reported in experimental studies where *Fusarium* species are common contaminants. As a result, FB_1 contamination of foodstuffs, particularly in maize and maize-based products may be considered a high risk factor for oesophageal cancer and Idiopathic congestive Cardiopathy in Gauteng province of South Africa. However, more data and individual level studies will be needed to sustain this assumption.

Evaluating human exposure to FB_1 and the linked health effects by assessing specific biomarkers in faecal material is a new approach. Furthermore the use of bio-wipe kit to collect faecal material is a step forward approach considering the problem with collecting faecal material from rural areas. The use of bio-wipe kit has several advantages for assessing FB_1 intake since it is able to measure exposure at an individual level. Although FB_1 was successfully detected, drawbacks in this study were also observed particularly on the volume of faecal material gathered from the kit. The total volume gathered was insufficient, resulting in working with smaller volume of extraction solvents. Thus, to improve the sensitivity and accuracy of the method it is suggested that participants use several bio-wipes each time they go to the toilet. Also, this would be an interesting investigation as variation in the levels of FB_1 over a period could be done giving a more statistically accurate idea of exposure. The study indicates that in the study area, faecal material as a FB_1 biomarker would be a tool not only in the estimation of

exposure of a population of FB_1 but also in the assessment of intervention strategies to reduce FB_1 exposure, particularly maize and maize-based products are consumed as staple diet.

6.7 CONCLUSION

There are several reports from South Africa on contamination of *Fusarium* species particularly those belonging to *F. verticillioides* and *F. proliferatum* complex, widely distributed at high contamination levels in maize. In this study, it has been observed that *F. verticillioides* was the main contaminant in faecal material, resulting in increased prevalence and levels of FB_1 and spores of the fungus in faecal material samples. To reduce the high risks of exposure, biomarkers need to be developed particularly in developing countries. This study has proposed a desirable, more responsive technique to determine dietary exposure to FB_1 using low cost methods compared to the expensive clean-up methods. The procedure is suggested as a suitable method for the determination of FB_1 in faecal material samples. Thin layer chromatography was a useful preliminary screening procedure to confirm the presence of FB_1 in samples, while HPLC was performed to determine the levels of the toxin present in each sample. In addition, FB_1 detected in bio-wipe samples in this study have not reach critical levels as compared to daily exposure through consumption of contaminated food, which could lead to detrimental health effects on humans depending on the volume and the length of exposure. Accordingly, this is the first report to describe the use of bio-wipe kit as a rapid, cost effective technique and concluded that the bio-wipe kit is an appropriate method of faecal collection to determine the exposure to FB_1 in Gauteng Province, South Africa.

CHAPTER SEVEN

General Conclusion

This study was designed with the objectives to investigate the role in which houseflies play in the dissemination of fungal spores, to assess and quantify the toxigenic potentials of the fungi isolated, to assess the toxic effect of the fungal extracts on human mononuclear cells. To meet all the objectives houseflies, maize, porridge and water samples were collected from the rural areas of Gauteng Province, South Africa. However, this is due to inadequate data on the incidence of fungal species disseminated by houseflies and the dangers of contamination of food and water particularly with reference to the effect on the health of the rural community. Therefore, there is need to conduct studies that may provide useful information on the occurrence and spread of toxigenic fungi by houseflies. The three genera of mycotoxins producing filamentous fungi that are important in food commodities: *Aspergillus, Penicillium* and *Fusarium* were isolated in samples and that includes *A. flavus*, *A. niger*, *A. parasiticus*, *A. ochraceus*, *F. verticillioides*, *F. proliferatum*, *F. oxysporum*, *F. culmorum*, *F. graminearum*, *P. aurantiogriseum*, *P. brevicompactum*, *P. janthinellum* and *P. verrucosum*. The fungal extracts in this study confirmed the production of the major mycotoxins namely, AFs (AFB_1, AFB_2, AFG_1 and AFG_2), DON, FB_1, OTA and ZEA suggesting that the population in the rural community might not only be exposed to one mycotoxin but to a combination of mycotoxins in their systems. The *in vitro* study conducted on human mononuclear cells revealed that the *Aspergillus*, *Fusarium* and *Penicillium* extracts from samples were found to be toxic to the cells. The cytotoxicity results confirmed the sensitivity of the protocol used, even though it was not possible to deduce the biochemical mode of action of the extracts based on the reduction in cell viability induced by the extracts.

Furthermore, a correlation between the fungal spp. from houseflies, maize, porridge and water samples was observed with some samples having simultaneously more than one fungal spp. and one type of mycotoxin. Hence, this confirmed the hypothesis that houseflies are vectors for fungal spores which could be deposited on foods and feeds and hence cause the spread of mycotoxicoses with adverse public health impact. Mycotoxins cause significant health problems all over the world, especially in rural areas where nutrition of people is based on not appropriately produced and stored grains, and thus highly contaminated agricultural produce

(mainly maize). Human exposure to mycotoxins by cereals is widely investigated, however there is very few information about alternative ways of contamination. Thus, the study gives a very current and new aspect on the hygienic importance of the houseflies and their role in infections, the possibly way, how fungi isolated from the flies may start mycotoxin production.

Interaction between insects and fungi in stored food commodity could indirectly lead to increase in the production of fungal toxins (mycotoxins) since the insects may spread the spores of dangerous moulds (toxigenic fungi). The mycotoxins are health hazards to mankind. For example the mycotoxins known as aflatoxins, which is associated with maize has been found to affect children leading to deformity and mental retardation. Of great importance is the possible distribution of such harmful bacteria as *Salmonella*, *Streptococcus* and *Escherichia coli* by grain infesting insects. Normally, freshly harvested grains, even before being milled into flour, are already contaminated with a range of potentially determinative agents, particularly insects and fungi from field and storage facilities. Mycoflora such as *Aspergillus*, *Penicillium* and *Cladosporium* have been reported hidden in maize, maize-based products and water reported in this study can serve as nutrient sources for fly development. The storage fungi normally accompany or follow insect infestation.

Faecal materials were also among the samples obtained from Gauteng Province to assess the use of a bio-wipe kit as a rapid method to determine human exposure to FB_1. *Fusarium* contamination was investigated in faecal material samples. It was observed that *F. verticillioides* and *F. proliferatum* were the main contaminant. Therefore, their presence in the faecal materials resulted in increased prevalence and levels of the mycotoxin of great importance like FB_1 which is thought to have toxigenic properties. Consumption of maize and maize-based products with high levels of mycotoxins does not invariably produce instant reaction, however, a long term exposure, may lead to adverse chronic effects on the consumers. In the rural areas, maize is consumed from the first year of life; therefore, chronic effects can be due to accumulation of FB_1 in combination with other factors. Because FB_1 reduces the uptake of folate in different cell lines, fumonisin consumption has been implicated in connection with neural tube defects in human babies. Furthermore, the incidence of fumonisin and other mycotoxins should be further investigated in rural areas of Gauteng Province. Additionally, this study introduces a new

method for FB_1 detection in faecal materials and a well-suited biomarker to determine the exposure to FB_1.

In conclusion, contamination of foods and feeds by different types of fungal spp. and mycotoxins is major problem in Africa affecting the general health of the public. The safety of food supply is of global concern and requires the commitment of all countries. A major reason countries import and export food is to satisfy consumer demand. Foodborne illnesses may be linked to the consumption of foods whether grown and manufactured domestically or imported. Global food safety standards are required to ensure that food will not be injurious to health regardless of its origin. Data obtained from these studies show there is a need for research work to be conducted in rural areas concerning mycotoxins studies; however, less has been conducted so far in rural areas of Gauteng province. That is why there is an urgent need to focus attention on rural areas in South Africa.

Recommendations

Houseflies are of major public health concerns in developing countries, particularly in the rural community of Gauteng Province of South Africa. South Africa is among the developing countries whereby rural communities have issues related to sanitation and hygiene that have been given low priority in comparison to other development needs. Being an agricultural country, subsistence farming and dairy farming plays a pivotal role in the rural areas by providing maize, milk and farm animal manures. The dairies usually play a significant role in the breeding of flies due to poor hygiene conditions in the form of farm manures, poor disposal and open defecation places. All these places are recognized as potential feeding and breeding places of houseflies. Improving environmental sanitation and hygiene of dairies, for example by reduction of the breeding sites and sources that attract flies and provide effective housefly control. Since the housefly can complete its life cycle in as little as seven days, removal of wet manure is necessary to break the breeding cycle. Wet straw should not be allowed to pile up in or near buildings, since straw is one of the best fly breeding materials. Moreover, the installation and use of latrines with a concealed sewage system in such places can further minimize various diseases spreading due to the inability of flies to come in contact with pathogens and faeces. Another way could be by means of recycling and rebaiting of the fly traps used to during sample collection in this

study. With the outcome of this approach, the community can objectively control housefly population.

However, there is little or no information concerning community awareness of the problems associated with houseflies and their management. By increasing the awareness of hygiene and its role in suppressing the spread of houseflies and epidemics would increase the demands for latrines. Although knowledge of the problems associated with houseflies was not directly related with housefly prevention practices, yet this is an important one because unless people have the knowledge of hazards associated with flies they would not consider it a serious pest. As we understand that there is a direct association between the farmer's education level and knowledge of houseflies breeding sites, and housefly prevention practices, therefore, fixing the gap between knowledge, education and management practices will remain an important task for houseflies control in the future that would ultimately save many lives. Housefly is a very important vector of many harmful germs and so cause severe health problems particularly in Sub Saharan Africa. While many studies have given insight to the bacteria and viruses spread by this vector, there are truly very few studies on the fungal profile of the external body of the housefly. Therefore, this makes the work quite novel in Africa which carries the greatest burden of diseases.

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APPENDICES

Appendix I

Table 1.1 Fungal contamination on the external surfaces of female and male houseflies from Gauteng Province, South Africa.

		Fungal species and Fungal load			
SN	**SS**	**Females**		**Males**	
1	H	*A. carbonarius* *A. flavus* *C. herbarum* *F. equiseti* *F. proliferatum* *F. semitectum* *F. verticillioides*	3.8 x 10^5	*A. flavus* *A. parasiticus* *C. herbarum* *F. culmorum* *F. proliferatum* *F. verticillioides*	2.0 x 10^5
2	H	*A. flavus* *A. parasiticus* *F. oxysporum* *M. plumbeus* *P. crustosum* *P. sclerotiorum*	5.3 x 10^3	*A. clavatus* *A. ochraceus* *F. oxysporum* *F. proliferatum* *F. verticillioides* *M. plumbeus* *P. oslonii*	7.0 x 10^3
3	H	*A. flavus* *A. niger* *A. parasiticus* *A. infectoria* *F. avenaceum* *F. culmorum* *F. oxysporum* *F. proliferatum* *F. verticillioides* *P. verrucosum*	1.4 x 10^5	*A. carbonarius* *A. flavus* *A. niger* *A. parasiticus* *A. ochraceus* *A. infectoria* *C. herbarum* *F. culmorum* *F. proliferatum* *P. aurantiogriseum* *P. brevicompactum*	2.4 x 10^4
4	H	*A. carbonarius* *A. infectoria* *F. culmorum* *F. oxysporum* *F. proliferatum* *F. semitectum* *F. verticillioides* *M. racemosus* *P. janthinellum*	3.9 x 10^4	*A. niger* *C. herbarum* *C. lunata* *A. infectoria* *F. poae* *F. graminearum* *F. culmorum* *F. oxysporum* *F. proliferatum* *F. verticillioides* *P. crustosum*	4.2 x 10^4

SN: sample number; **SS**: sample site; **H**: House; **T**: Toilet.

Table 1.1 continued…….

SN	SS	Fungal species and Fungal load			
		Females		Males	
5	H	*A. flavus* *A. parasiticus* *M. suaveolens* *P. verrucosum* *S. brevicaulis*	1.8×10^6	-	-
6	H	*A. carbonarius* *A. flavus* *A. parasiticus* *M. suaveolens*	6.0×10^6	*A. flavus* *A. niger* *M. suaveolens* *S. brevicaulis*	3.8×10^5
7	H	*A. niger* *A. infectoria* *F. poae* *F. culmorum* *F. nivale* *F. oxysporum* *P. brevicompactum* *P. verrucosum*	7.0×10^3	*A. carbonarius* *A. flavus* *A. parasiticus* *A. infectoria* *C. lunata* *F. avenaceum* *P. brevicompactum*	7.1×10^4
8	H	*A. carbonarius* *A. flavus* *A. fumigatus* *A. niger* *A. parasiticus* *F. proliferatum* *F. verticillioides* *M. suaveolens*	6.4×10^1	*A. flavus* *A. parasiticus* *F. culmorum* *M. suaveolens* *P. verrucosum*	4.6×10^1
9	H	*A. flavus* *A. niger* *F. culmorum* *F. oxysporum* *F. proliferatum* *F. sporotrichioides*	2.1×10^4	*A. carbonarius* *A. clavatus* *A. niger* *A. parasiticus* *P. aurantiogriseum*	1.1×10^3
10	H	*F. verticillioides*	1.5×10^7	*F. verticillioides* *F. poae*	1.5×10^7
11	H	*A. niger* *A. ochraceus* *P. brevicompactum*	6.3×10^4	*A. fumigatus* *A. parasiticus* *F. oxysporum* *M. suaveolens* *P. oslonii*	1.2×10^4

SN: sample number; **SS**: sample site; **H**: House; **T**: Toilet.

Table 1.1 continued…….

SN	SS	Fungal species and Fungal load			
		Females		**Males**	
12	H	*A. flavus* *A. fumigatus* *A. parasiticus* *A. ochraceus* *F. oxysporum* *F. verticillioides* *P. oslonii* *P. verrucosum*	2.0 x 10^5	*A. candidus* *A. carbonarius* *A. flavus* *A. niger* *A. parasiticus* *A. ustus* *A. wentii* *F. avenaceum* *P. crustosum* *P. aurantiogriseum* *P. janthinellum* *M. suaveolens*	2.7 x 10^6
13	H	*A. carbonarius* *A. ustus* *P. brevicompactum* *P. janthinellum* *P. verrucosum*	7.7 x 10^6	*A. carbonarius* *A. flavus* *A. ustus* *C. herbarum* *M. racemosus* *P. verrucosum*	5.2 x 10^6
14	H	*A. flavus* *A. fumigatus* *A. niger* *A. ochraceus* *A. parasiticus* *A. ustus* *F. verticillioides* *P. crustosum* *P. janthinellum*	7.5 x 10^5	*F. proliferatum* *M. suaveolens* *M. plumbeus*	4.8 x 10^8
15	H	*A. clavatus* *A. flavus* *A. fumigatus* *A. niger* *A. parasiticus* *F. sporotrichioides* *F. semitectum* *F. nivale* *F. proliferatum* *F. verticillioides* *M. plumbeus* *P. oslonii*	1.0 x 10^7	-	-

SN: sample number; **SS**: sample site; **H**: House; **T**: Toilet.

Table 1.1 continued…….

SN	SS	Fungal species and Fungal load			
		Females		Males	
16	T	*A. niger*	2.8×10^7	-	-
17	H	*A. niger* *A. oryzae* *A. parasiticus* *P. aurantiogriseum* *P. brevicompactum* *P. janthinellum*	5.8×10^6	*A. carbonarius* *M. plumbeus* *P. janthinellum*	4.0×10^5
18	H	*A. flavus* *F. graminearum* *R. microspores*	1.5×10^7	*P. crustosum*	1.1×10^3
19	H	*A. flavus* *A. niger* *F. verticillioides* *P. citrinum* *P. crustosum*	4.7×10^4	-	-
20	H	*A. flavus*	1.5×10^7	-	-
21	H	*A. flavus* *C. paralopsis* *F. proliferatum* *F. sporotrichioides* *P. aurantiogriseum* *P. janthinellum* *P. verrucosum*	1.5×10^7	*A. flavus* *A. ustus* *F. equiseti* *F. avenaceum* *F. oxysporum* *F. proliferatum* *F. culmorum* *F. verticillioides* *P. brevicompactum* *P. verrucosum*	3.1×10^3
22	H	*A. clavatus* *A. flavus* *A. parasiticus* *A. ochraceus* *F. equiseti* *F. verticillioides* *P. verrucosum*	7.8×10^5	*A. flavus* *A. fumigatus* *A. parasiticus* *P. citrinum* *F. verticillioides*	1.1×10^5
23	T	*A. flavus* *A. fumigatus* *P. aurantiogriseum* *P. brevicompactum*	1.1×10^6	-	-

SN: sample number; **SS**: sample site; **H**: House; **T**: Toilet.

Table 1.1 continued…….

SN	SS	Fungal species and Fungal load			
		Females		Males	
24	H	*A. flavus* *C. krusei* *C. inops* *C. herbarum* *E. nigrum* *M. suaveolens*	1.5×10^7	*M. suaveolens*	1.5×10^7
25	H	*A. flavus*	4.0×10^6	*A. flavus* *S. brevicaulis*	3.0×10^6
26	T	*F. verticillioides* *C. inops*	1.5×10^7	-	-
27	T	*A. oryzae* *C. krusei* *M. suaveolens*	3.2×10^6	-	-
28	H	*A. clavatus* *A. flavus* *M. racemosus*	5.0×10^5	*A. flavus* *M. suaveolens*	1.5×10^7
29	H	*A. flavus* *A. niger* *C. fornicola* *F. poae* *F. proliferatum* *F. oxysporum* *F. verticillioides* *R. microspores* *R. oligosporus*	8.4×10^4	*A. flavus* *A. fumigatus*	3.0×10^4
30	H	*A. parasiticus* *A. oryzae* *M. piriformis*	2.1×10^5	-	-
31	H	*A. flavus* *P. aurantiogriseum* *P. expansum* *P. oslonii* *R. microspores*	2.0×10^5	*A. flavus* *P. brevicompactum* *P. crustosum*	3.5×10^3
32	H	*A. clavatus* *C. krusei* *P. aurantiogriseum* *R. microspores*	1.4×10^6	-	-

SN: sample number; **SS**: sample site; **H**: House; **T**: Toilet.

Table 1.1 continued…….

SN	SS	Fungal species and Fungal load			
		Females		Males	
33	H	*C. krusei* *F. graminearum*	1.6 x 10^6	*A. flavus* *A. infectoria* *E. nigrum* *F. verticillioides* *P. aurantiogriseum* *P. verrucosum*	5.4 x 10^3
34	H	*A. flavus* *A. niger* *P. aurantiogriseum* *R. oryzae* *R. microspores*	1.5 x 10^7	*A. flavus* *R. stolonifer*	3.4 x 10^5
35	H	*C. krusei* *E. nigrum* *M. suaveolens*	2.3 x 10^6	-	-
36	H	*A. flavus* *C. krusei* *M. suaveolens*	1.4 x 10^7	-	-
37	H	*A. flavus* *M. suaveolens* *E. nigrum*	1.3 x 10^6	-	-
38	H	*A. flavus* *E. nigrum*	1.4 x 10^6	-	-
39	H	*A. flavus* *M. piriformis*	5.7 x 10^5	-	-
40	H	*A. ochraceus* *F. proliferatum*	4.7 x 10^4	-	-
41	T	*A. flavus* *E. nigrum* *P. aurantiogriseum* *M. suaveolens*	1.3 x 10^6	-	-
42	H	*A. ochraceus* *M. suaveolens* *P. crustosum* *P. janthinellum* *P. oslonii*	4.1 x 10^5	-	-

SN: sample number; **SS**: sample site; **H**: House; **T**: Toilet.

Table 1.1 continued…….

SN	SS	Fungal species and Fungal load			
		Females		Males	
43	T	*A. flavus* *A. niger* *C. krusei*	4.8 x 10^4	-	-
44	H	*C. krusei*	3.0 x 10^4	-	-
45	H	*A. carbonarius* *C. krusei* *M. suaveolens*	1.5 x 10^7	-	-
46	H	*A. ustus* *C. herbarum*	2.6 x 10^7	-	-
47	H	*A. flavus* *C. krusei* *P. aurantiogriseum*	5.7 x 10^4	-	-
48	H	*A. ochraceus* *M. suaveolens* *P. verrucosum*	1.5 x 10^6	-	-
49	T	*A. flavus* *S. brevicaulis*	1.2 x 10^6	-	-
50	H	*A. flavus* *A. parasiticus*	1.5 x 10^7	*S. brevicaulis*	1.1 x 10^7
51	H	*M. suaveolens* *S. brevicaulis* *P. citrinum* *P. crustosum*	2.0 x 10^3	-	-
52	T	*M. suaveolens*	1.2 x 10^7	-	-
53	H	*A. flavus* *A. parasiticus* *A. ochraceus* *C. fornicola* *P. brevicompactum*	1.5 x 10^7	-	-
54	H	*A. flavus* *C. paralopsis* *C. fornicola* *P. aurantiogriseum* *P. brevicompactum* *P. janthinellum*	1.4 x 10^7	-	-

SN: sample number; **SS**: sample site; **H**: House; **T**: Toilet.

Table 1.1 continued…….

SN	SS	Fungal species and Fungal load			
		Females		Males	
55	H	*A. flavus* *A. niger* *A. ochraceus* *P. janthinellum* *P. verrucosum* *M. suaveolens*	7.9 x 10^6	*A. carbonarius* *A. ochraceus* *C. fornicola* *P. janthinellum* *P. verrucosum*	8.0 x 10^6
56	H	*A. ochraceus* *M. suaveolens* *P. janthinellum*	1.5 x 10^7	-	-
57	H	*A. flavus* *A. niger* *P. oslonii* *P. verrucosum*	1.5 x 10^7	-	-
58	H	*A. niger* *M. suaveolens*	7.8 x 10^6	-	-
59	H	*F. proliferatum* *F. verticillioides* *P. aurantiogriseum* *P. brevicompactum*	2.0 x 10^7	*C. paralopsis* *M. suaveolens* *P. verrucosum*	1.6 x 10^7
60	H	*A. flavus* *A. ustus*	2.1 x 10^5	-	-
61	H	*A. flavus* *M. suaveolens* *P. aurantiogriseum* *P. brevicompactum* *P. verrucosum*	9.5 x 10^4	-	-
62	H	*A. flavus* *A. niger* *C. fornicola* *M. suaveolens*	4.0 x 10^6	-	-
63	H	*A. niger* *F. proliferatum* *F. verticillioides* *M. suaveolens* *P. brevicompactum*	4.2 x 10^5	-	-
64	H	*M. suaveolens*	1.2 x 10^6	-	-

SN: sample number; **SS**: sample site; **H**: House; **T**: Toilet.

Table 1.1 continued…….

SN	SS	Fungal species and Fungal load			
		Females		**Males**	
65	H	*A. ochraceus* *A. niger* *M. suaveolens* *P. oslonii* *P. verrucosum*	3.3 x 10^6	*A. flavus* *P. janthinellum* *P. oslonii* *P. verrucosum*	5.0 x 10^6
66	H	*M. suaveolens* *P. verrucosum*	1.5 x 10^7		
67	H	*E. javanicum* *M. suaveolens* *P. brevicompactum*	6.0 x 10^6	*A. fumigatus* *M. suaveolens* *P. brevicompactum*	3.8 x 10^5
68	H	*E. javanicum* *P. brevicompactum* *P. verrucosum*	2.7 x 10^6	-	-
69	T	*M. suaveolens* *P. janthinellum*	1.5 x 10^7	-	-
70	H	*E. javanicum* *S. brevicaulis*	7.4 x 10^6	-	-
71	T	*A. niger* *E. javanicum*	4.1 x 10^3	-	-
72	T	*A. carbonarius* *A. flavus* *E. javanicum* *P. aurantiogriseum*	1.5 x 10^7	-	-
73	T	*A. ochraceus* *P. aurantiogriseum* *P. crustosum*	8.0 x 10^3	-	-
74	H	*A. niger* *C. fornicola* *P. janthinellum* *P. verrucosum* *S. brevicaulis*	1.5 x 10^7	*A. ustus* *A. wentii* *C. fornicola* *M. suaveolens* *P. aurantiogriseum* *P. citrinum*	7.0 x 10^6
75	H	*A. fumigatus* *A. ustus* *E. nigrum* *E. javanicum* *M. piriformis*	5.2 x 10^4	-	-

SN: sample number; **SS**: sample site; **H**: House; **T**: Toilet.

Table 1.1 continued…….

SN	SS	Fungal species and Fungal load			
		Females		Males	
76	H	*A. carbonarius* *A. flavus* *A. ustus* *P. crustosum* *P. verrucosum*	8.5 x 10^6	*A. flavus* *A. ustus*	4.1 x 10^6
77	H	*A. carbonarius* *A. flavus* *A. niger* *M. suaveolens* *P. citrinum* *S. brevicaulis*	3.0 x 10^4	-	-
78	H	*F. proliferatum* *M. suaveolens*	1.5 x 10^7	*A. flavus* *C. herbarum* *E. nigrum* *P. aurantiogriseum* *M. suaveolens* *R. microspores*	2.2 x 10^4
79	H	*A. flavus* *A. ochraceus* *P. aurantiogriseum* *M. suaveolens*	7.8 x 10^4	*A. carbonarius* *A. flavus* *A. fumigatus* *F. poae*	2.2 x 10^4
80	H	*A. niger* *F. oxysporum* *F. verticillioides* *P. brevicompactum* *P. verrucosum* *S. brevicaulis*	1.2 x 10^6	*A. flavus* *A. fumigatus*	1.1 x 10^2
81	H	*A. flavus* *M. suaveolens*	3.3 x 10^5	*A. flavus* *M. suaveolens*	7.1 x 10^4
82	H	*A. flavus* *A. niger* *M. suaveolens*	2.1 x 10^5	*A. flavus* *M. suaveolens*	3.0 x 10^2
83	H	*A. flavus* *M. suaveolens*	1.5 x 10^7	*A. flavus* *A. parasiticus* *P. aurantiogriseum*	1.2 x 10^3
84	H	*M. suaveolens* *P. aurantiogriseum* *P. crustosum*	3.4 x 10^5	*A. ochraceus* *M. suaveolens* *P. oslonii*	1.5 x 10^5

SN: sample number; **SS**: sample site; **H**: House; **T**: Toilet.

Table 1.2 Fungal contamination in maize from Gauteng Province, South Africa

Sample no.	Sample I.D	Fungal name	Fungal load (CFU/g[a])
1	DB1	*C. cladosporium* *F. culmorum* *F. nivale* *F. oxysporum* *F. verticillioides* *M. suaveloens*	2×10^5
2	DB2	*C. cladosporioides* *F. avenaceum* *F. culmorum* *F. nivale* *F. oxysporum* *F. proliferatum* *F. verticillioides* *M. suaveloens* *P. oslonii* *R. microsporus*	1×10^6
3	DB3	*A. carbonarius* *C. cladosporioides* *F. dimerum* *F. proliferatum* *F. verticillioides* *M. plumbeus* *P. oslonii*	4×10^6
4	DB5	*A. flavus* *A. ochraceus* *C. cladosporioides* *F. avenaceum* *F. oxysporum* *F. proliferatum* *F. verticillioides* *M. plumbeus* *P. janthinellum* *P. expansum* *P. oslonii* *S. brevicaulis*	8×10^5

[a]**CFU/g: Colony forming unit per gram**

Table 1.2 Continued……

Sample no.	Sample I.D	Fungal name	Fungal load (CFU/g[a])
5	DB6	*F. avenaceum* *F. culmorum* *F. oxysporum* *F. proliferatum* *F. verticillioides* *P. citrinum* *P. oslonii* *S. brevicaulis* *R. microsporus*	1×10^6
6	DB8	*F. acuminatum* *F. oxysporum* *F. verticillioides* *P. aurantiogriseum* *P. brevicompactum*	8×10^6
7	DB9	*A. carbonarius* *A. flavus* *A. niger* *A. parasiticus* *F. culmorum* *F. graminearum* *F. nivale* *F. oxysporum* *F. semitectum* *F. verticillioides* *M. sauveolens* *P. aurantiogriseum* *P. citrinum* *P. crustosum* *P. expansum* *P. janthinellum* *P. verrucosum*	2×10^6
8	DB11	*F. oxysporum* *F. verticillioides* *P. citrinum* *P. oslonii* *R. microsporus*	4×10^6

[a]CFU/g: Colony forming unit per gram.

Table 1.2 Continued……

Sample no.	Sample I.D	Fungal name	Fungal load (CFU/g[a])
9	J1	*A. carbonarius* *A. flavus* *A. niger* *A. parasiticus* *C. cladosporioides* *F. culmorum* *F. nivale* *F. oxysporum* *F. proliferatum* *F. semitectum* *F. verticillioides* *P. aurantiogriseum* *P. brevicompactum* *P. citrinum* *P. crustosum* *P. expansum* *P. oslonii*	3×10^6
10	J5	*A. carbonarius* *A. flavus* *F. culmorum* *F. sporotrichioides* *F. verticillioides* *P. oslonii* *P. verrucosum*	4×10^6
11	J9	*C. cladosporioides* *A. parasiticus* *F. avenaceum* *F. culmorum* *F. graminearum* *F. nivale* *F. oxysporum* *F. verticillioides* *M. sauveolens* *P. expansum* *P. janthinellum* *P. oslonii* *S. brevecaulis*	5×10^5

[a]**CFU/g: Colony forming unit per gram.**

Table 1.2 Continued……

Sample no.	Sample I.D	Fungal name	Fungal load (CFU/g[a])
12	J17	*A. flavus* *A. niger* *A. parasiticus* *F. culmorum* *F. graminearum* *F. nivale* *F. oxysporum* *F. proliferatum* *F. verticillioides* *P. citrinum* *P. oslonii*	1×10^5
13	J19	*F. culmorum* *F. graminearum* *F. subglutinans* *F. verticillioides* *P. aurantiogriseum* *P. brevicompactum* *P. oslonii* *P. verrucosum*	2×10^6
14	J20	*A. flavus* *A. parasiticus* *F. acuminatum* *F. oxysporum* *F. proliferatum* *F. sporotrichioides* *M. plumbeus* *P. citrinum* *P. oslonii* *P. verrucosum*	4×10^5
15	J21	*C. cladosporioides* *F. graminearum* *F. verticillioides* *P. aurantiogriseum* *P. citrinum* *P. expansum* *P. janthinellum* *P. oslonii* *M. sauveolens*	2×10^6

[a]**CFU/g: Colony forming unit per gram.**

Table 1.3 Fungal contamination in porridge from Gauteng Province, South Africa

Sample no.	Sample I.D	Fungal name	Fungal load (CFU/g[a])
1	DB1	*A. carbonarius*	2×10^6
		A. flavus	
		A. niger	
		A. ochraceus	
		A. parasiticus	
		C. krusei	
2	DB3	*P. janthinellum*	4×10^6
3	DB5	*M. plumbeus*	1×10^7
4	DB6	*A. niger*	5×10^4
		P. verrucosum	
5	DB8	*M. sauveolens*	5×10^5
6	DB9	*M. plembeus*	1×10^5
7	DB10	*P. oslonii*	9×10^5
8	DB11	*A. parasiticus*	1×10^7
		M. sauveolens	
		P. oslonii	
9	J3	*A. flavus*	7×10^4
		C. herbarum	
		M. plembeus	
		M. sauveolens	
10	J5	*A. flavus*	5×10^6
		A. parasiticus	
11	J12A	*A. flavus*	1×10^7
		A. parasiticus	
		M. plumbeus	
12	J12B	*C. herbarum*	2×10^5
		M. sauveolens	
13	J17	*C. herbarum*	7×10^3
		A. flavus	
14	J19	*A. parasiticus*	2×10^6
		A. verrucosum	
15	J20	*A. niger*	3×10^6
		C. herbarum	
16	J21	*A. niger*	4×10^5
		M. sauveolens	
		P. citrinum	
17	J22	*M. plumbeus*	6×10^5
		M. sauveolens	
18	J23	*A. flavus*	10×10^4
19	J24	*C. herbarum*	3×10^4

[a]**CFU/g: Colony forming unit per gram.**

Table 1.3 Fungal contamination in water from Gauteng Province, South Africa

Sample no.	Sample I.D	Fungal name	Fungal load (CFU/g[a])
1	DB1	*A. flavus*	4 x 10^1
		A. parasiticus	
2	DB2	*A. niger*	12 x 10^1
		A. parasiticus	
3	DB3	*P. verrucosum*	1 x 10^1
4	DB5	*P. oslonii*	1 x 10^1
5	DB6	*A. niger*	1 x 10^1
6	DB8	*A. niger*	9 x 10^1
		A. parasiticus	
		P. oslonii	
7	DB9	*A. flavus*	15 x 10^1
		A. parasiticus	
8	DB10	*A. niger*	8 x 10^1
		P. oslonii	
9	DB11	*A. flavus*	2 x 10^1
		A. niger	
10	DB12	*A. niger*	1 x 10^1
11	J12A	*A. flavus*	4 x 10^1
		A. parasiticus	
		M. plumbeus	
12	J1	*A. parasiticus*	28 x 10^1
13	J2	*A. flavus*	2 x 10^1
14	J3	*A. niger*	3 x 10^1
15	J5	*A. niger*	2 x 10^1
		P. verrucosum	
16	J6	*A. flavus*	1 x 10^1
		A. niger	
17	J9	*A. niger*	2 x 10^1
18	J12	*A. niger*	22 x 10^1
19	J12A	*A. flavus*	2 x 10^1
		C. herbarum	
20	J15	*A. niger*	2 x 10^1
		P. oslonii	
21	J17	*A. flavus*	2 x 10^1
		A. niger	
22	J19	*A. niger*	2 x 10^1
23	J20	*A. niger*	2 x 10^1
24	J21	*A. niger*	1 x 10^1
25	J22	*A. flavus*	2 x 10^1
		A. parasiticus	
		P. oslonii	
26	J23	*A. flavus*	12 x 10^1
		A. parasiticus	
27	J24	*A. parasiticus*	5 x 10^1
		A. niger	

[a]CFU/g: Colony forming unit per gram.

Appendix II

Table 2.1 Production of Aflatoxins and Ochratoxin A by *Aspergillus* extracts in houseflies from Gauteng Province, South Africa.

Sample ID	Sample name	AFB_1	AFB_2	AFG_1	AFG_2	OTA
$1HF_1$	*A. carbonarius*	0	0	0	0	158
$1HF_2$	*A. flavus*	69	35	0	0	0
$1HM_1$	*A. parasiticus*	4	0	0	0	0
$1HM_2$	*A. flavus*	3880	12	0	0	0
$2HF_1$	*A. flavus*	221	87	0	0	0
$2HF_3$	*A. parasiticus*	1562	146	78	49	0
$2HM_1$	*A. clavatus*	0	0	0	0	0
$2HM_6$	*A. ochraceus*	0	0	0	0	198
$3HF_1$	*A. parasiticus*	9	6	0	0	0
$3HF_2$	*A. flavus*	114	19	0	0	0
$3HF_7$	*A. niger*	0	0	0	0	36
$3HM_1$	*A. parasiticus*	665	19	10	0	0
$3HM_2$	*A. flavus*	49	18	0	0	0
$3HM_3$	*A. carbonarius*	0	0	0	0	399
$3HM_4$	*A. ochraceus*	0	0	0	0	2561
$3HM_{10}$	*A. niger*	0	0	0	0	298
$4HF_1$	*A. carbonarius*	0	0	0	0	411
$4HM_1$	*A. niger*	0	0	0	0	183
$5HF_1$	*A. flavus*	3015	99	0	0	0
$5HF_2$	*A. parasiticus*	523	89	0	0	0
$6HF_1$	*A. flavus*	5016	44	0	0	0
$6HF_2$	*A. parasiticus*	145	124	35	4	0
$6HF_3$	*A. carbonarius*	0	0	0	0	701
$6HM_1$	*A. flavus*	647	101	0	0	0
$6HM_2$	*A. niger*	0	0	0	0	189
$7HF_1$	*A. niger*	0	0	0	0	1642
$7HM_1$	*A. parasiticus*	29	0	0	0	0
$7HM_2$	*A. carbonarius*	0	0	0	0	68
$7HM_4$	*A. flavus*	398	31	0	0	0
$8HF_1$	*A. niger*	0	0	0	0	219
$8HF_2$	*A. carbonarius*	0	0	0	0	69
$8HF_3$	*A. fumigatus*	0	0	0	0	0
$8HF_4$	*A. parasiticus*	101	12	3	0	0
$8HF_5$	*A. flavus*	1004	148	0	0	0
$8HM_1$	*A. flavus*	1119	58	0	0	0

Note: AFB_1: Aflatoxin B_1, AFB_2: Aflatoxin B_2, AFG_1: Aflatoxin G_1, AFG_2: Aflatoxin G_2 and OTA: Ochratoxin A. Concentrations of mycotoxins produced are recorded in µg/kg.

Table 2.1 Continued……

Sample ID	Sample name	AFB_1	AFB_2	AFG_1	AFG_2	OTA
$8HM_2$	*A. parasiticus*	1670	27	9	6	0
$9HF_1$	*A. flavus*	621	57	0	0	0
$9HF_2$	*A. niger*	0	0	0	0	167
$9HM_1$	*A. clavatus*	0	0	0	0	0
$9HM_2$	*A. niger*	0	0	0	0	633
$9HM_3$	*A. carbonarius*	0	0	0	0	61
$11HM_4$	*A. flavus*	1021	38	0	0	0
$11HF_2$	*A. ochraceus*	0	0	0	0	790
$11HF_3$	*A. niger*	0	0	0	0	89
$11HM_1$	*A. fumigatus*	0	0	0	0	0
$11HM_2$	*A. parasiticus*	336	4	0	0	0
$12HF_1$	*A. flavus*	68	12	0	0	0
$12HF_2$	*A. fumigatus*	0	0	0	0	0
$12HF_3$	*A. ochraceus*	0	0	0	0	1221
$12HF_4$	*A. parasiticus*	2251	135	113	0	0
$12HM_1$	*A. niger*	0	0	0	0	538
$12HM_2$	*A. carbonarius*	0	0	0	0	113
$12HM_3$	*A. flavus*	20	18	0	0	0
$12HM_4$	*A. wentii*	0	0	0	0	0
$12HM_5$	*A. ustus*	0	0	0	0	0
$12HM_6$	*A. parasiticus*	0	0	0	0	0
$12HM_{13}$	*A. candidus*	0	0	0	0	0
$13HF_1$	*A. carbonarius*	0	0	0	0	109
$13HF_2$	*A. ustus*	0	0	0	0	0
$13HM_1$	*A. carbonarius*	0	0	0	0	69
$13HM_2$	*A. flavus*	46	34	0	0	0
$13HM_4$	*A. ustus*	0	0	0	0	0
$14HF_1$	*A. flavus*	69	29	0	0	0
$14HF_2$	*A. parasiticus*	20	0	0	0	0
$14HF_3$	*A. fumigatus*	0	0	0	0	0
$14HF_4$	*A. ochraceus*	0	0	0	0	1020
$14HF_5$	*A. niger*	0	0	0	0	228
$14HF_{10}$	*A. ustus*	0	0	0	0	0
$15HF_1$	*A. parasiticus*	48	28	0	0	0
$15HF_2$	*A. flavus*	38	26	0	0	0
$15HF_3$	*A. niger*	0	0	0	0	307
$15HF_4$	*A. clavatus*	0	0	0	0	0
$15HF_5$	*A. fumigatus*	0	0	0	0	0
$16HF_1$	*A. niger*	0	0	0	0	267

Note: AFB_1: Aflatoxin B_1, AFB_2: Aflatoxin B_2, AFG_1: Aflatoxin G_1, AFG_2: Aflatoxin G_2 and OTA: Ochratoxin A. Concentrations of mycotoxins produced are recorded in µg/kg.

Table 2.1 Continued……

Sample ID	Sample name	AFB_1	AFB_2	AFG_1	AFG_2	OTA
$17HF_1$	*A. oryzae*	0	0	0	0	0
$17HF_2$	*A. parasiticus*	24	0	0	0	0
$17HF_3$	*A. niger*	0	0	0	0	126
$17HM_1$	*A. carbonarius*	0	0	0	0	38
$18HF_1$	*A. flavus*	86	21	0	0	0
$19HF_4$	*A. flavus*	5	0	0	0	0
$19HF_5$	*A. niger*	0	0	0	0	525
$20HF_1$	*A. flavus*	0	0	0	0	0
$21HF_3$	*A. flavus*	0	0	0	0	0
$21HM_5$	*A. ustus*	0	0	0	0	0
$21HM_6$	*A. flavus*	0	0	0	0	0
$22HF_3$	*A. ochraceus*	0	0	0	0	128
$22HF_4$	*A. flavus*	10	0	0	0	0
$22HF_5$	*A. parasiticus*	0	0	0	0	0
$22HF_8$	*A. clavatus*	0	0	0	0	0
$22HM_2$	*A. fumigatus*	0	0	0	0	0
$22HM_3$	*A. parasiticus*	0	0	0	0	0
$22HM_4$	*A. flavus*	11	0	0	0	0
$23HF_1$	*A. flavus*	2346	149	0	0	0
$23HF_3$	*A. fumigatus*	0	0	0	0	0
$24HF_4$	*A. flavus*	0	0	0	0	0
$25HF_1$	*A. flavus*	0	0	0	0	0
$25HM_1$	*A. flavus*	0	0	0	0	0
$27HF_1$	*A. oryzae*	0	0	0	0	0
$28HF_1$	*A. clavatus*	0	0	0	0	0
$28HF_2$	*A. flavus*	0	0	0	0	0
$28HM_1$	*A. flavus*	9	0	0	0	0
$29HF_1$	*A. flavus*	4	0	0	0	0
$29HF_2$	*A. niger*	0	0	0	0	369
$29HM_1$	*A. flavus*	7	7	0	0	0
$29HM_2$	*A. fumigatus*	0	0	0	0	0
$30HF_1$	*A. parasiticus*	0	0	0	0	0
$30HF_2$	*A. oryzae*	0	0	0	0	0
$31HF_4$	*A. flavus*	10	4	0	0	0
$31HM_4$	*A. flavus*	0	0	0	0	0
$32HF_1$	*A. clavatus*	0	0	0	0	0
$34HF_2$	*A. flavus*	0	0	0	0	0
$34HF_3$	*A. niger*	0	0	0	0	489

Note: AFB_1: Aflatoxin B_1, AFB_2: Aflatoxin B_2, AFG_1: Aflatoxin G_1, AFG_2: Aflatoxin G_2 and OTA: Ochratoxin A. Concentrations of mycotoxins produced are recorded in µg/kg.

Table 2.1 Continued……

Sample ID	Sample name	AFB_1	AFB_2	AFG_1	AFG_2	OTA
$34HM_1$	*A. flavus*	5014	138	0	0	0
$36HF_1$	*A. flavus*	0	0	0	0	0
$37HF_1$	*A. flavus*	0	0	0	0	0
$38HF_4$	*A. flavus*	516	0	0	0	0
$39HF_1$	*A. flavus*	9	0	0	0	0
$40HF_1$	*A. ochraceus*	0	0	0	0	214
$41HF_2$	*A. flavus*	31	9	0	0	0
$42HF_4$	*A. ochraceus*	0	0	0	0	115
$43HF_3$	*A. flavus*	14	0	0	0	0
$43HF_4$	*A. niger*	0	0	0	0	269
$45HF_1$	*A. carbonarius*	0	0	0	0	113
$46HF_2$	*A. ustus*	0	0	0	0	0
$47HF_2$	*A. flavus*	0	0	0	0	0
$48HF_3$	*A. ochraceus*	0	0	0	0	0
$49HF_1$	*A. flavus*	101	38	0	0	0
$50HF_1$	*A. flavus*	315	62	0	0	0
$50HF_2$	*A. parasiticus*	149	51	28	0	0
$53HF_1$	*A. parasiticus*	0	0	0	0	0
$53HF_4$	*A. flavus*	0	0	0	0	0
$53HF_5$	*A. ochraceus*	0	0	0	0	433
$54HF_4$	*A. flavus*	31	11	0	0	0
$55HF_1$	*A. flavus*	19	0	0	0	0
$55HF_4$	*A. ochraceus*	0	0	0	0	81
$55HF_5$	*A. niger*	0	0	0	0	63
$55HM_1$	*A. carbonarius*	0	0	0	0	98
$55HM_4$	*A. ochraceus*	0	0	0	0	302
$56HF_1$	*A. ochraceus*	0	0	0	0	33
$57HF_4$	*A. flavus*	11	0	0	0	0
$57HF_5$	*A. niger*	0	0	0	0	35
$58HF_2$	*A. niger*	0	0	0	0	29
$60HF_1$	*A. flavus*	144	0	0	0	0
$60HF_2$	*A. ustus*	0	0	0	0	0
$61HF_1$	*A. flavus*	56	0	0	0	0
$62HF_1$	*A. flavus*	3101	0	0	0	0
$62HF_2$	*A. niger*	0	0	0	0	18
$63HF_2$	*A. niger*	0	0	0	0	0
$65HF_3$	*A. ochraceus*	0	0	0	0	236
$65HF_4$	*A. niger*	0	0	0	0	52
$65HM_4$	*A. flavus*	44	9	0	0	0

Note: AFB_1: Aflatoxin B_1, AFB_2: Aflatoxin B_2, AFG_1: Aflatoxin G_1, AFG_2: Aflatoxin G_2 and OTA: Ochratoxin A. Concentrations of mycotoxins produced are recorded in µg/kg.

Table 2.1 Continued……

Sample ID	Sample name	AFB_1	AFB_2	AFG_1	AFG_2	OTA
$67HM_2$	*A. fumigatus*	0	0	0	0	0
$71HF_1$	*A. niger*	0	0	0	0	236
$72HF_1$	*A. carbonarius*	0	0	0	0	429
$72HF_2$	*A. flavus*	31	29	0	0	0
$73HF_3$	*A. ochraceus*	0	0	0	0	698
$74HF_6$	*A. niger*	0	0	0	0	348
$74HM_1$	*A. ustus*	0	0	0	0	0
$74HM_2$	*A. wentii*	0	0	0	0	0
$75HF_1$	*A. ustus*	0	0	0	0	0
$75HF_2$	*A. fumigatus*	0	0	0	0	0
$76HF_1$	*A. flavus*	308	115	0	0	0
$76HF_2$	*A. carbonarius*	0	0	0	0	119
$76HF_3$	*A. ustus*	0	0	0	0	0
$76HM_1$	*A. flavus*	389	88	0	0	0
$76HM_2$	*A. ustus*	0	0	0	0	0
$77HF_1$	*A. carbonarius*	0	0	0	0	1301
$77HF_2$	*A. niger*	0	0	0	0	157
$77HF_3$	*A. flavus*	69	37	0	0	0
$78HM_1$	*A. flavus*	7	5	0	0	0
$79HF_1$	*A. flavus*	21	7	0	0	0
$79HF_2$	*A. ochraceus*	0	0	0	0	684
$79HM_1$	*A. flavus*	89	18	0	0	0
$79HM_2$	*A. fumigatus*	0	0	0	0	0
$79HM_3$	*A. carbonarius*	0	0	0	0	37
$80HF_4$	*A. niger*	0	0	0	0	52
$80HM_1$	*A. fumigatus*	0	0	0	0	0
$80HM_2$	*A. flavus*	77	68	0	0	0
$81HF_1$	*A. flavus*	0	0	0	0	0
$81HM_1$	*A. flavus*	0	0	0	0	0
$82HF_1$	*A. flavus*	0	0	0	0	0
$82HF_2$	*A. niger*	0	0	0	0	0
$82HM_1$	*A. flavus*	927	91	0	0	0
$83HF_1$	*A. flavus*	3	0	0	0	0
$83HM_1$	*A. parasiticus*	1625	183	0	0	0
$83HM_2$	*A. flavus*	54	0	0	0	0
$84HM_1$	*A. ochraceus*	0	0	0	0	0

Note: AFB_1: Aflatoxin B_1, AFB_2: Aflatoxin B_2, AFG_1: Aflatoxin G_1, AFG_2: Aflatoxin G_2 and OTA: Ochratoxin A. Concentrations of mycotoxins produced are recorded in µg/kg.

Table 2.2 Production of Ochratoxin A by *Penicillium* extracts in houseflies from Gauteng Province, South Africa.

Sample ID	Sample name	AFB_1	AFB_2	AFG_1	AFG_2	OTA
$2HF_2$	*P. crustosum*	0	0	0	0	0
$2HF_4$	*P. sclerotiorum*	0	0	0	0	0
$2HM_2$	*P. oslonii*	0	0	0	0	0
$3HF_7$	*P. verrucosum*	0	0	0	0	715
$3HM_5$	*P. aurantiogriseum*	0	0	0	0	0
$3HM_6$	*P. brevicompactum*	0	0	0	0	0
$4HM_2$	*P. crustosum*	0	0	0	0	0
$4HF_3$	*P. janthinellum*	0	0	0	0	0
$5HF_3$	*P. verrucosum*	0	0	0	0	527
$7HF_2$	*P. brevicompactum*	0	0	0	0	0
$7HF_3$	*P. verrucosum*	0	0	0	0	1268
$7HM_5$	*P. brevicompactum*	0	0	0	0	0
$8HM_3$	*P. verrucosum*	0	0	0	0	623
$9HM_5$	*P. aurantiogriseum*	0	0	0	0	0
$11HM_1$	*P. oslonii*	0	0	0	0	519
$11HF_1$	*P. brevicompactum*	0	0	0	0	0
$12HF_5$	*P. verrucosum*	0	0	0	0	299
$12HF_6$	*P. oslonii*	0	0	0	0	0
$12HM_8$	*P. aurantiogriseum*	0	0	0	0	0
$12HM_9$	*P. janthinellum*	0	0	0	0	0
$12HM_{10}$	*P. crustosum*	0	0	0	0	0
$13HF_3$	*P. verrucosum*	0	0	0	0	1789
$13HF_4$	*P. janthinellum*	0	0	0	0	0
$13HF_5$	*P. brevicompactum*	0	0	0	0	0
$13HM_7$	*P. verrucosum*	0	0	0	0	0
$14HF_8$	*P. crustosum*	0	0	0	0	0
$14HF_9$	*P. janthinellum*	0	0	0	0	0
$15HF_6$	*P. oslonii*	0	0	0	0	0
$17HF_4$	*P. janthinellum*	0	0	0	0	0
$17HF_5$	*P. brevicompactum*	0	0	0	0	0
$17HF_6$	*P. aurantiogriseum*	0	0	0	0	0
$17HM_2$	*P. janthinellum*	0	0	0	0	0
$18HM_2$	*P. crustosum*	0	0	0	0	0
$19HF_3$	*P. crustosum*	0	0	0	0	0
$19HF_7$	*P. citrinum*	0	0	0	0	0
$21HF_1$	*P. janthinellum*	0	0	0	0	0
$21HF_2$	*P. verrucosum*	0	0	0	0	291
$21HF_7$	*P. aurantiogriseum*	0	0	0	0	0

Note: AFB_1: Aflatoxin B_1, AFB_2: Aflatoxin B_2, AFG_1: Aflatoxin G_1, AFG_2: Aflatoxin G_2 and OTA: Ochratoxin A. Concentrations of mycotoxins produced are recorded in µg/kg.

Table 2.2 Continued……

Sample ID	Sample name	AFB_1	AFB_2	AFG_1	AFG_2	OTA
$21HM_2$	*P. verrucosum*	0	0	0	0	1034
$21HM_3$	*P. brevicompactum*	0	0	0	0	0
$22HF_6$	*P. verrucosum*	0	0	0	0	1318
$22HM_1$	*P. citrinum*	0	0	0	0	0
$23HF_1$	*P. aurantiogriseum*	0	0	0	0	0
$23HF_2$	*P. brevicompactum*	0	0	0	0	0
$31HF_1$	*P. expansum*	0	0	0	0	0
$31HF_2$	*P. oslonii*	0	0	0	0	0
$31HF_3$	*P. aurantiogriseum*	0	0	0	0	0
$31HM_2$	*P. brevicompactum*	0	0	0	0	0
$31HM_3$	*P. crustosum*	0	0	0	0	0
$33HM_4$	*P. verrucosum*	0	0	0	0	914
$32HM_8$	*P. aurantiogriseum*	0	0	0	0	0
$34HF_1$	*P. aurantiogriseum*	0	0	0	0	0
$33HF_2$	*P. aurantiogriseum*	0	0	0	0	0
$41HF_1$	*P. aurantiogriseum*	0	0	0	0	0
$42HF_1$	*P. janthinellum*	0	0	0	0	0
$42HF_2$	*P. crustosum*	0	0	0	0	0
$42HF_3$	*P. oslonii*	0	0	0	0	0
$47HF_1$	*P. aurantiogriseum*	0	0	0	0	0
$48HF_3$	*P. verrucosum*	0	0	0	0	29
$51HF_1$	*P. citrinum*	0	0	0	0	0
$51HF_2$	*P. crustosum*	0	0	0	0	0
$53HF_2$	*P. brevicompactum*	0	0	0	0	0
$54HF_1$	*P. janthinellum*	0	0	0	0	0
$54HF_2$	*P. aurantiogriseum*	0	0	0	0	0
$54HF_5$	*P. brevicompactum*	0	0	0	0	0
$55HF_2$	*P. janthinellum*	0	0	0	0	0
$55HF_3$	*P. verrucosum*	0	0	0	0	125
$55HM_2$	*P. verrucosum*	0	0	0	0	1548
$56HM_3$	*P. janthinellum*	0	0	0	0	0
$57HF_2$	*P. verrucosum*	0	0	0	0	1994
$57HF_3$	*P. oslonii*	0	0	0	0	0
$59HF_1$	*P. aurantiogriseum*	0	0	0	0	0
$59HF_2$	*P. brevicompactum*	0	0	0	0	0
$59HM_3$	*P. verrucosum*	0	0	0	0	278
$61HF_2$	*P. aurantiogriseum*	0	0	0	0	0
$61HF_4$	*P. verrucosum*	0	0	0	0	459

Note: AFB_1: Aflatoxin B_1, AFB_2: Aflatoxin B_2, AFG_1: Aflatoxin G_1, AFG_2: Aflatoxin G_2 and OTA: Ochratoxin A. Concentrations of mycotoxins produced are recorded in µg/kg.

Table 2.2 Continued……..

Sample ID	Sample name	AFB_1	AFB_2	AFG_1	AFG_2	OTA
$61HF_1$	*P. brevicompactum*	0	0	0	0	0
$63HF_1$	*P. brevicompactum*	0	0	0	0	0
$65HF_1$	*P. verrucosum*	0	0	0	0	654
$65HF_2$	*P. oslonii*	0	0	0	0	0
$65HM_1$	*P. janthinellum*	0	0	0	0	0
$65HM_2$	*P. verrucosum*	0	0	0	0	1169
$65HM_3$	*P. oslonii*	0	0	0	0	0
$66HF_2$	*P. verrucosum*	0	0	0	0	1082
$67HF_1$	*P. brevicompactum*	0	0	0	0	0
$67HM_1$	*P. brevicompactum*	0	0	0	0	0
$68HF_1$	*P. brevicompactum*	0	0	0	0	0
$68HF_2$	*P. verrucosum*	0	0	0	0	272
$69HF_1$	*P. janthinellum*	0	0	0	0	0
$72HF_3$	*P. aurantiogriseum*	0	0	0	0	0
$73HF_1$	*P. crustosum*	0	0	0	0	0
$73HF_2$	*P. aurantiogriseum*	0	0	0	0	0
$74HF_4$	*P. janthinellum*	0	0	0	0	0
$74HF_5$	*P. verrucosum*	0	0	0	0	108
$74HM_3$	*P. oslonii*	0	0	0	0	0
$74HM_4$	*P. aurantiogriseum*	0	0	0	0	0
$76HF_4$	*P. crustosum*	0	0	0	0	0
$76HF_5$	*P. verrucosum*	0	0	0	0	1764
$77HF_4$	*P. citrinum*	0	0	0	0	0
$78HM_2$	*P. aurantiogriseum*	0	0	0	0	0
$79HF_3$	*P. aurantiogriseum*	0	0	0	0	0
$80HF_5$	*P. brevicompactum*	0	0	0	0	0
$83HM_3$	*P. aurantiogriseum*	0	0	0	0	0
$84HF_1$	*P. crustosum*	0	0	0	0	0
$84HF_2$	*P. aurantiogriseum*	0	0	0	0	0
$84HM_2$	*P. oslonii*	0	0	0	0	0

Note: AFB_1: Aflatoxin B_1, AFB_2: Aflatoxin B_2, AFG_1: Aflatoxin G_1, AFG_2: Aflatoxin G_2 and OTA: Ochratoxin A. Concentrations of mycotoxins produced are recorded in µg/kg.

Table 2.3 Production of DON, FB_1 and ZEA by *Fusarium* extracts in houseflies from Gauteng Province, South Africa.

Sample ID	Sample name	DON	FB_1	ZEA
$1HF_7$	*F. verticillioides*	0	1816	0
$1HF_8$	*F. proliferatum*	0	162	0
$1HF_{10}$	*F. equiseti*	0	0	117
$1HF_{11}$	*F. semitectum*	0	0	156
$1HM_3$	*F. verticillioides*	0	1064	0
$1HM_6$	*F. culmorum*	6	0	1
$1HM_7$	*F. proliferatum*	0	147	0
$2HF_6$	*F. oxysporum*	0	0	918
$2HM_5$	*F. proliferatum*	0	0	0
$2HM_7$	*F. oxysporum*	0	0	49
$2HM_8$	*F. verticillioides*	0	631	0
$3HF_4$	*F. culmorum*	0	0	0
$3HF_8$	*F. avenaceum*	0	0	0
$3HF_{12}$	*F. oxysporum*	0	0	18
$3HF_9$	*F. verticillioides*	0	187	0
$3HF_{10}$	*F. proliferatum*	0	131	0
$3HM_{11}$	*F. proliferatum*	0	182	0
$3HM_{13}$	*F. culmorum*	0	0	41
$4HF_7$	*F. verticillioides*	0	1144	0
$4HF_9$	*F. proliferatum*	0	140	0
$4HF_{10}$	*F. semitectum*	0	0	55
$4HF_{11}$	*F. culmorum*	2	0	1263
$4HF_{12}$	*F. oxysporum*	0	0	39
$4HM_1$	*F. graminearum*	2	0	758
$4HM_8$	*F. culmorum*	3	0	37
$4HM_9$	*F. poae*	2	0	0
$4HM_{10}$	*F. oxysporum*	0	0	48
$4HM_{11}$	*F. proliferatum*	0	172	0
$4HM_{12}$	*F. verticillioides*	0	240	0
$7HF_6$	*F. culmorum*	5	0	34
$7HF_7$	*F. poae*	3	0	0
$7HF_8$	*F. nivale*	0	0	0
$7HF_9$	*F. oxysporum*	0	0	103
$7HM_9$	*F. avenaceum*	0	0	0
$8HF_9$	*F. verticillioides*	0	1001	0
$8HF_7$	*F. proliferatum*	0	198	0
$8HM_7$	*F. culmorum*	0	0	325

Note: DON: Deoxynivalenol, FB_1: Fumonisin B_1 and ZEA: Zearalenone. Concentrations of mycotoxins produced are recorded in µg/kg.

Table 2.3 Continued……..

Sample ID	Sample name	DON	FB_1	ZEA
$9HF_4$	*F. sporotrichioides*	2	0	0
$9HF_5$	*F. oxysporum*	0	0	25
$9HF_6$	*F. culmorum*	0	0	338
$9HM_4$	*F. proliferatum*	0	198	0
$10HF_1$	*F. verticillioides*	0	94	0
$10HM_1$	*F. poae*	0	0	0
$10HM_2$	*F. verticillioides*	0	52	0
$11HM_4$	*F. oxysporum*	0	0	18
$12HM_8$	*F. oxysporum*	0	0	21
$12HM_9$	*F. verticillioides*	0	113	0
$12HM_{12}$	*F. avenaceum*	0	0	0
$14HF_6$	*F. verticillioides*	0	114	0
$14HM_7$	*F. verticillioides*	0	12	0
$14HM_1$	*F. proliferatum*	0	84	0
$15HF_7$	*F. sporotrichioides*	3	0	0
$15HF_8$	*F. verticillioides*	0	108	0
$15HF_9$	*F. nivale*	0	0	0
$15HF_{11}$	*F. semitectum*	0	0	30
$15HF_{12}$	*F. proliferatum*	0	159	0
$18HF_2$	*F. graminearum*	4	0	2
$19HF_1$	*F. verticillioides*	0	1067	0
$21HF_4$	*F. sporotrichioides*	0	0	0
$21HF_5$	*F. proliferatum*	0	118	0
$21HM_5$	*F. verticillioides*	0	126	0
$21HM_7$	*F. proliferatum*	0	249	0
$21HM_8$	*F. equiseti*	0	0	0
$21HM_9$	*F. avenaceum*	0	0	0
$21HM_{10}$	*F. oxysporum*	0	0	37
$21HM_{11}$	*F. culmorum*	2	0	24
$22HM_1$	*F. verticillioides*	0	0	0
$22HF_2$	*F. verticillioides*	0	361	0
$26HF_7$	*F. verticillioides*	0	266	0
$29HF_5$	*F. poae*	3	0	0
$29HF_6$	*F. oxysporum*	0	0	4
$29HF_7$	*F. proliferatum*	0	93	0
$29HF_8$	*F. verticillioides*	0	99	0
$33HF_6$	*F. graminearum*	1	0	37
$33HM_7$	*F. verticillioides*	0	16	0
$40HF_2$	*F. proliferatum*	0	224	0

Note: DON: Deoxynivalenol, FB_1: Fumonisin B_1 and ZEA: Zearalenone. Concentrations of mycotoxins produced are recorded in µg/kg.

Table 2.3 Continued……..

Sample ID	Sample name	DON	FB_1	ZEA
$59HF_4$	*F. proliferatum*	0	0	0
$59HF_5$	*F. verticillioides*	0	168	0
$63HF_4$	*F. verticillioides*	0	141	0
$63HF_5$	*F. proliferatum*	0	136	0
$78HF_1$	*F. proliferatum*	0	144	0
$79HM_4$	*F. poae*	1	0	0
$80HF_1$	*F. verticillioides*	0	167	0
$80HF_5$	*F. oxysporum*	0	0	11

Note: DON: Deoxynivalenol, FB_1: Fumonisin B_1 and ZEA: Zearalenone. Concentrations of mycotoxins produced are recorded in µg/kg.

Table 2.4 Production of Aflatoxins and Ochratoxin A by *Aspergillus* extracts in maize from Gauteng Province, South Africa.

Sample ID	Sample name	AFB_1	AFB_2	AFG_1	AFG_2	OTA
$3M_1$	*A. carbonarius*	0	0	0	0	2945
$4M_5$	*A. flavus*	9859	4977	0	0	0
$4M_6$	*A. ochraceus*	0	0	0	0	2810
$7M_4$	*A. flavus*	2165	74	0	0	0
$7M_5$	*A. carbonarius*	0	0	0	0	2631
$7M_6$	*A. parasiticus*	4112	3047	328	0	0
$7M_7$	*A. niger*	0	0	0	0	1258
$9M_{14}$	*A. parasiticus*	8541	2081	735	0	0
$9M_5$	*A. flavus*	3045	2299	0	0	0
$9M_{16}$	*A. niger*	0	0	0	0	1518
$9M_{17}$	*A. carbonarius*	0	0	0	0	669
$10M_4$	*A. flavus*	3152	0	0	0	0
$10M_5$	*A. carbonarius*	0	0	0	0	1647
$11M_{10}$	*A. parasiticus*	1350	174	107	0	0
$12M_{10}$	*A. flavus*	2120	1289	0	0	0
$12M_{11}$	*A. parasiticus*	55	47	0	0	0
$12M_{12}$	*A. niger*	0	0	0	0	4
$14M_5$	*A. parasiticus*	2891	50	0	0	0
$14M_6$	*A. flavus*	17988	5025	0	0	0

Note: AFB_1: Aflatoxin B_1, AFB_2: Aflatoxin B_2, AFG_1: Aflatoxin G_1, AFG_2: Aflatoxin G_2 and OTA: Ochratoxin A. Concentrations of mycotoxins produced are recorded in µg/kg.

Table 2.5 Production of Aflatoxins and Ochratoxin A by *Penicillium* extracts in maize from Gauteng Province, South Africa.

Sample ID	Sample name	AFB_1	AFB_2	AFG_1	AFG_2	OTA
$2M_7$	*P. oslonii*	0	0	0	0	0
$3M_2$	*P. oslonii*	0	0	0	0	0
$4M_7$	*P. expansum*	0	0	0	0	0
$4M_8$	*P. oslonii*	0	0	0	0	0
$4M_9$	*P. janthinellum*	0	0	0	0	0
$5M_6$	*P. citrinum*	0	0	0	0	0
$5M_7$	*P. oslonii*	0	0	0	0	0
$6M_3$	*P. brevicompactum*	0	0	0	0	0
$6M_4$	*P. aurantiogriseum*	0	0	0	0	0
$7M_7$	*P. citrinum*	0	0	0	0	0
$7M_8$	*P. expansum*	0	0	0	0	0
$7M_{14}$	*P. janthinellum*	0	0	0	0	0
$7M_{15}$	*P. crustosum*	0	0	0	0	0
$7M_{16}$	*P. aurantiogriseum*	0	0	0	0	0
$7M_{17}$	*P. verrucosum*	0	0	0	0	4
$8M_3$	*P. oslonii*	0	0	0	0	0
$8M_4$	*P. citrinum*	0	0	0	0	0
$9M_8$	*P. expansum*	0	0	0	0	0
$9M_9$	*P. citrinum*	0	0	0	0	0
$9M_{10}$	*P. oslonii*	0	0	0	0	0
$9M_{11}$	*P. brevicompactum*	0	0	0	0	0
$9M_{12}$	*P. aurantiogriseum*	0	0	0	0	0
$10M_6$	*P. oslonii*	0	0	0	0	0
$10M_7$	*P. verrucosum*	0	0	0	0	13
$11M_7$	*P. expansum*	0	0	0	0	0
$11M_8$	*P. oslonii*	0	0	0	0	0
$11M_9$	*P. janthinellum*	0	0	0	0	0
$12M_8$	*P. oslonii*	0	0	0	0	0
$12M_9$	*P. citrinum*	0	0	0	0	0
$13M_6$	*P. verrucosum*	0	0	0	0	0
$13M_7$	*P. oslonii*	0	0	0	0	0
$13M_8$	*P. aurantiogriseum*	0	0	0	0	0
$13M_9$	*P. brevicompactum*	0	0	0	0	0
$13M_{10}$	*P. verrucosum*	0	0	0	0	0
$14M_7$	*P. citrinum*	0	0	0	0	0
$14M_8$	*P. oslonii*	0	0	0	0	0
$14M_9$	*P. verrucosum*	0	0	0	0	6
$15M_3$	*P. janthinellum*	0	0	0	0	0
$15M_4$	*P. citrinum*	0	0	0	0	0
$15M_5$	*P. aurantiogriseum*	0	0	0	0	0
$15M_6$	*P. expansum*	0	0	0	0	0
$15M_7$	*P. oslonii*	0	0	0	0	0

Note: AFB_1: Aflatoxin B_1, AFB_2: Aflatoxin B_2, AFG_1: Aflatoxin G_1, AFG_2: Aflatoxin G_2 and OTA: Ochratoxin A. Concentrations of mycotoxins produced are recorded in µg/kg.

Table 2.6 Production of DON, FB_1 and ZEA by *Fusarium* extracts from maize.

Sample ID	Sample name	DON	FB_1	ZEA
$1M_1$	*F. culmorum*	1	0	2
$1M_2$	*F. verticillioides*	0	1859	0
$1M_3$	*F. nivale*	0	0	0
$1M_4$	*F. oxysporum*	0	0	3
$2M_1$	*F. culmorum*	5	0	6
$2M_2$	*F. oxysporum*	0	0	3
$2M_3$	*F. verticillioides*	0	342	0
$2M_4$	*F. proliferatum*	0	221	0
$2M_5$	*F. nivale*	0	0	0
$2M_6$	*F. avenaceum*	0	0	0
$3M_1$	*F. proliferatum*	0	236	0
$3M_2$	*F. verticillioides*	0	3920	0
$3M_3$	*F. culmorum*	0	0	2
$4M_1$	*F. proliferatum*	0	176	0
$4M_2$	*F. oxysporum*	0	0	1
$4M_3$	*F. verticillioides*	0	304	0
$4M_4$	*F. avenaceum*	0	0	0
$5M_1$	*F. culmorum*	2	0	5
$5M_2$	*F. proliferatum*	0	144	0
$5M_3$	*F. verticillioides*	0	319	0
$5M_4$	*F. oxysporum*	0	0	3
$5M_5$	*F. avenaceum*	0	0	0
$6M_1$	*F. verticillioides*	0	18	0
$6M_2$	*F. oxysporum*	0	0	4
$6M_5$	*F. culmorum*	1	0	9
$7M_1$	*F. nivale*	0	15	0
$7M_2$	*F. graminearum*	2	0	19
$7M_3$	*F. oxysporum*	0	0	16
$7M_4$	*F. semitectum*	0	0	6
$7M_5$	*F. verticillioides*	0	66	0
$8M_1$	*F. oxysporum*	0	0	4
$8M_2$	*F. semitectum*	0	0	3
$9M_1$	*F. verticillioides*	0	7	0
$9M_2$	*F. culmorum*	4	0	5
$9M_3$	*F. culmorum*	1	0	2
$9M_4$	*F. verticillioides*	0	539	0
$9M_5$	*F. oxysporum*	0	0	8

Note: DON: Deoxynivalenol, FB_1: Fumonisin B_1 and ZEA: Zearalenone. Concentrations of mycotoxins produced are recorded in µg/kg.

Table 2.6 Continued……..

Sample ID	Sample name	DON	FB_1	ZEA
$9M_6$	*F. nivale*	0	0	0
$9M_7$	*F. graminearum*	0	0	4
$10M_1$	*F. avenaceum*	0	0	0
$10M_2$	*F. graminearum*	5	0	11
$10M_3$	*F. verticillioides*	0	1654	0
$11M_1$	*F. proliferatum*	0	32	0
$11M_2$	*F. nivale*	0	0	0
$11M_3$	*F. graminearum*	2	0	3
$11M_4$	*F. oxysporum*	0	0	0
$11M_5$	*F. culmorum*	5	0	11
$11M_6$	*F. graminearum*	2	0	1
$12M_1$	*F. verticillioides*	0	412	0
$12M_2$	*F. oxysporum*	0	0	7
$12M_3$	*F. semitectum*	0	0	0
$12M_4$	*F. culmorum*	4	0	6
$12M_5$	*F. nivale*	0	0	0
$12M_6$	*F. proliferatum*	0	18	0
$12M_7$	*F. sporotrichioides*	8	0	0
$13M_1$	*F. verticillioides*	0	495	0
$13M_2$	*F. culmorum*	5	0	0
$13M_3$	*F. sporotrichioides*	5	0	0
$13M_4$	*F. graminearum*	2	0	14
$13M_5$	*F. sporotrichioides*	2	0	18
$14M_1$	*F. oxysporum*	0	0	15
$14M_2$	*F. proliferatum*	0	3	0
$14M_3$	*F. verticillioides*	0	21	0
$14M_4$	*F. graminearum*	4	0	3
$15M_1$	*F. verticillioides*	0	17	0

Note: DON: deoxynivalenol, FB_1: fumonisin B_1 and ZEA: zearalenone. Concentrations of mycotoxins produced are recorded in µg/kg.

Table 2.7 Production of Aflatoxins and Ochratoxin A by *Aspergillus* and *Penicillium* extracts in porridge from Gauteng Province, South Africa.

Sample ID	Sample name	AFB_1	AFB_2	AFG_1	AFG_2	OTA
$1P_1$	*A. parasiticus*	2847	2390	895	798	0
$1P_2$	*A. carbonarius*	0	0	0	0	17
$1P_3$	*A. niger*	0	0	0	0	0.3
$1P_4$	*A. flavus*	5294	4911	0	0	0
$1P_5$	*A. ochraceus*	0	0	0	0	6
$4P_1$	*A. flavus*	1068	0	0	0	0
$4P_3$	*A. niger*	0	0	0	0	50
$8P_1$	*A. parasiticus*	7299	2587	0	0	0
$9P_1$	*A. flavus*	3891	2512	0	0	0
$10P_1$	*A. flavus*	1417	201	0	0	0
$10P_2$	*A. parasiticus*	2425	1234	0	0	0
$11P_1$	*A. parasiticus*	2168	824	489	0	0
$11P_2$	*A. flavus*	1113	0	0	0	0
$13P_2$	*A. flavus*	1396	1136	0	0	0
$14P_1$	*A. parasiticus*	4742	632	0	0	0
$15P_2$	*A. niger*	0	0	0	0	31
$16P_3$	*A. niger*	0	0	0	0	20
$18P_1$	*A. flavus*	2032	633	0	0	0
$2P_1$	*P. janthinellum*	0	0	0	0	0
$4P_2$	*P. verrucosum*	0	0	0	0	60
$6P_3$	*P. aurantiogriseum*	0	0	0	0	0
$7P_2$	*P. oslonii*	0	0	0	0	0
$8P_2$	*P. oslonii*	0	0	0	0	0
$14P_2$	*P. verrucosum*	0	0	0	0	0
$14P_3$	*P. citrinum*	0	0	0	0	0

Note: AFB_1: Aflatoxin B_1, AFB_2: Aflatoxin B_2, AFG_1: Aflatoxin G_1, AFG_2: Aflatoxin G_2 and OTA: Ochratoxin A. Concentrations of mycotoxins produced are recorded in µg/kg.

Table 2.8 Toxigenicity of Aflatoxins and Ochratoxin A by *Aspergillus* and *Penicillium* extracts in water from Gauteng Province, South Africa.

Sample ID	Sample name	AFB_1	AFB_2	AFG_1	AFG_2	OTA
$1W_1$	*A. flavus*	1827	743	0	0	0
$1W_2$	*A. parasiticus*	4596	3321	131	0	0
$2W_1$	*A. niger*	0	0	0	0	347
$2W_2$	*A. parasiticus*	7974	7642	709	0	0
$2W_3$	*A. flavus*	1053	739	0	0	0
$5W_1$	*A. niger*	0	0	0	0	1238
$6W_1$	*A. parasiticus*	2607	586	534	0	0
$6W_2$	*A. niger*	0	0	0	0	540
$7W_1$	*A. parasiticus*	2200	359	158	0	0
$7W_2$	*A. flavus*	426	410	0	0	0
$7W_3$	*A. parasiticus*	329	0	0	0	0
$8W_1$	*A. niger*	0	0	0	0	4
$9W_1$	*A. niger*	0	0	0	0	747
$9W_2$	*A. flavus*	148	128	0	0	0
$10W_1$	*A. niger*	0	0	0	0	1738
$11W_1$	*A. parasiticus*	3731	3	0	0	0
$12W_1$	*A. flavus*	714	234	0	0	0
$13W_1$	*A. niger*	0	0	0	0	0.3
$14W_1$	*A. niger*	0	0	0	0	10
$15W_1$	*A. flavus*	4997	0	0	0	0
$15W_2$	*A. niger*	0	0	0	0	10
$16W_1$	*A. niger*	0	0	0	0	7
$17W_1$	*A. niger*	0	0	0	0	6
$18W_1$	*A. flavus*	840	452	0	0	0
$19W_1$	*A. niger*	0	0	0	0	894
$20W_1$	*A. niger*	0	0	0	0	5000
$21W_1$	*A. flavus*	4716	277	0	0	0
$21W_2$	*A. niger*	0	0	0	0	6
$22W_1$	*A. niger*	0	0	0	0	10
$23W_1$	*A. niger*	0	0	0	0	9
$24W_1$	*A. niger*	0	0	0	0	10
$25W_1$	*A. flavus*	5713	2108	0	0	0
$25W_2$	*A. parasiticus*	2491	1737	1195	101	0
$26W_1$	*A. flavus*	4078	1179	0	0	0
$26W_2$	*A. parasiticus*	5902	1481	0	0	0
$27W_1$	*A. parasiticus*	1482	1187	0	0	0
$27W_2$	*A. niger*	0	0	0	0	80
$3W_1$	*P. verrucossum*	0	0	0	0	8
$4W_1$	*P. oslonii*	0	0	0	0	0
$6W_1$	*P. oslonii*	0	0	0	0	0
$8W_1$	*P. oslonii*	0	0	0	0	0
$14W_1$	*P. verrucosum*	0	0	0	0	0
$20W_1$	*P. oslonii*	0	0	0	0	0
$25W_1$	*P. oslonii*	0	0	0	0	0

Note: AFB_1: Aflatoxin B_1, AFB_2: Aflatoxin B_2, AFG_1: Aflatoxin G_1, AFG_2: Aflatoxin G_2 and OTA: Ochratoxin A. Concentrations of mycotoxins produced are recorded in µg/kg.

Appendix III

Table 3.1 Toxic effects of *Aspergillus* and *Penicillium* extracts from houseflies on human mononuclear cells over 24, 48 and 72hrs of exposure.

	% Cell viability								
	24hrs			48hrs			72hrs		
Extracts conc. µg/kg	**20 µl/ml**	**40 µl/ml**	**80 µl/ml**	**20 µl/ml**	**40 µl/ml**	**80 µl/ml**	**20 µl/ml**	**40 µl/ml**	**80 µl/ml**
A. flavus									
80	96	93	89	86	81	78	74	69	65
133	90	86	83	79	74	71	66	63	58
429	88	83	79	74	65	61	55	48	42
3114	86	81	75	71	65	61	55	48	42
5060	80	73	67	62	54	47	40	35	30
A. parasiticus									
76	94	90	87	83	80	76	72	69	66
340	90	87	83	80	76	71	68	64	59
694	88	84	80	76	74	70	65	61	57
1735	86	82	76	70	64	61	56	52	47
2499	84	79	72	65	60	56	51	46	40
A. carbonarius									
68	98	96	93	89	86	84	80	77	72
158	96	93	89	85	82	79	75	72	68
399	93	90	87	83	78	73	70	65	60
701	90	86	82	78	75	71	67	61	56
1301	89	85	81	76	70	65	60	54	48
A. niger									
52	98	95	91	88	84	81	78	74	70
189	96	94	90	85	82	78	73	70	67
298	94	91	88	84	80	76	72	68	64
525	90	87	82	85	80	76	70	66	61
1642	88	84	80	78	73	68	61	54	48
A. ochraceus									
33	98	96	94	92	88	85	83	79	75
214	94	90	86	83	79	75	72	67	61
790	90	86	82	79	74	71	68	62	57
1020	86	83	79	75	70	64	60	55	49
2561	82	78	73	68	62	58	53	48	42
P. verrucosum									
29	99	96	93	90	87	83	80	76	72
125	97	94	91	87	84	80	76	72	67
299	95	91	87	82	78	74	68	64	60
914	94	89	85	80	76	71	65	60	55
1764	90	96	91	86	80	75	69	63	57

Percentage of cell viability is recorded in triplicates.

Table 3.2 Toxic effects of *Fusarium* extracts from houseflies on human mononuclear cells over 24, 48 and 72hrs of exposure.

	% Cell viability								
	24hrs			48hrs			72hrs		
Extracts conc. µg/kg	**20 µl/ml**	**40 µl/ml**	**80 µl/ml**	**20 µl/ml**	**40 µl/ml**	**80 µl/ml**	**20 µl/ml**	**40 µl/ml**	**80 µl/ml**
F. culmorum									
7	98	96	93	89	86	81	77	74	70
26	95	92	89	85	83	80	76	73	69
41	96	94	90	87	82	78	75	71	66
325	94	91	87	84	80	77	71	66	60
1265	90	85	81	73	73	69	65	61	56
F. equiseti									
117	95	92	88	85	81	78	73	69	65
F. graminearum									
6	98	96	93	89	86	83	79	75	71
38	96	93	89	86	83	79	76	72	69
760	92	88	85	81	78	74	69	65	59
F. oxysporum									
4	98	96	94	90	87	84	81	78	74
11	96	93	90	86	82	79	76	73	70
39	98	96	92	88	83	79	75	71	68
103	95	92	86	83	80	78	74	71	67
918	94	90	86	83	78	74	69	63	57
F. poae									
1	99	97	95	92	90	87	85	82	79
3	98	96	93	91	88	85	82	76	72
3	97	96	92	89	87	85	81	78	75
F. proliferatum									
84	99	96	92	88	85	81	78	73	68
118	97	93	90	86	82	79	74	70	66
140	96	92	89	85	82	79	75	70	64
182	97	94	90	87	83	79	74	70	65
224	96	92	89	85	82	77	72	67	63
F. semitectum									
30	98	96	92	88	85	81	78	75	71
55	96	93	89	86	81	77	73	70	67
156	97	94	91	87	83	78	73	69	66
F. sporotrichioides									
2	99	97	94	91	87	83	80	77	73
3	98	95	93	89	87	84	81	78	72
F. verticillioides									
16	98	95	91	87	84	80	77	73	70
52	97	94	90	85	82	78	74	71	68
361	95	91	88	83	79	76	72	67	62
1064	92	88	83	79	75	70	66	61	57
1816	90	86	81	76	72	67	61	56	50

Percentage of cell viability is recorded in triplicates.

Table 3.3 Toxic effects of *Aspergillus* and *Penicillium* extracts from maize on human mononuclear cells over 24, 48 and 72hrs of exposure.

	% Cell viability								
	24hrs			48hrs			72hrs		
Extracts conc. µg/kg	**20 µl/ml**	**40 µl/ml**	**80 µl/ml**	**20 µl/ml**	**40 µl/ml**	**80 µl/ml**	**20 µl/ml**	**40 µl/ml**	**80 µl/ml**
A. flavus									
2239	89	84	80	77	73	70	66	61	55
3409	84	80	77	73	69	64	59	53	47
5344	80	76	72	68	64	59	54	48	40
14836	81	75	70	66	61	55	50	42	35
23013	79	72	67	61	56	49	44	35	27
A. parasiticus									
102	96	93	90	86	82	79	75	70	64
1631	90	87	83	80	75	72	67	62	54
2941	88	83	77	72	67	63	58	52	45
7487	84	78	73	69	63	59	53	47	40
11357	80	75	69	64	60	56	52	46	39
A. carbonarius									
669	89	85	82	77	73	69	64	60	56
1647	87	82	78	76	72	67	62	57	49
2631	84	80	75	71	65	61	57	51	46
2945	82	77	71	66	62	57	51	46	42
A. niger									
4	97	94	90	87	84	80	77	73	69
1518	86	82	77	71	66	62	58	54	49
1258	89	84	78	73	68	64	62	56	52
A. ochraceus									
2810	82	77	73	67	63	57	53	49	44
P. verrucosum									
6	99	96	93	89	86	82	79	75	70
13	98	94	90	85	81	76	73	69	65

Percentage of cell viability is recorded in triplicates.

Table 3.4 Toxic effects of *Fusarium* extracts from maize on human mononuclear cells over 24, 48 and 72hrs of exposure.

	% Cell viability								
	24hrs			48hrs			72hrs		
Extracts conc. µg/kg	**20 µl/ml**	**40 µl/ml**	**80 µl/ml**	**20 µl/ml**	**40 µl/ml**	**80 µl/ml**	**20 µl/ml**	**40 µl/ml**	**80 µl/ml**
F. culmorum									
3	99	97	94	92	89	86	82	79	76
3	99	96	94	91	88	84	82	79	75
5	99	96	93	90	86	83	80	76	73
10	98	96	93	89	86	82	79	76	72
15	98	95	91	87	85	81	78	74	71
F. graminearum									
4	99	97	94	90	87	83	81	78	74
7	99	97	94	91	87	84	81	79	76
16	97	95	91	87	85	81	78	74	71
16	98	95	92	88	85	81	78	75	72
21	98	95	92	88	85	82	79	75	72
F. oxysporum									
1	99	98	95	92	89	87	84	81	78
3	99	96	94	91	88	83	81	79	75
8	98	96	93	91	87	83	80	78	76
15	98	94	91	87	83	80	78	75	71
16	98	95	91	86	83	80	74	70	67
F. proliferatum									
3	98	96	93	90	87	84	80	76	72
32	97	95	92	88	83	79	75	72	70
144	95	92	88	84	79	75	72	67	64
176	96	92	89	84	79	73	69	66	62
236	94	90	85	81	78	74	69	66	61
F. semitectum									
3	98	96	92	90	87	84	81	75	71
6	98	96	93	89	85	82	77	73	70
F. sporotrichioides									
2	99	97	94	91	89	86	84	81	77
5	98	95	92	88	85	82	78	75	71
8	97	94	91	86	82	79	75	72	68
F. verticillioides									
18	98	96	92	89	85	81	78	74	69
304	95	92	88	83	79	76	72	67	63
342	96	92	89	84	80	75	71	66	62
1859	91	87	84	79	74	68	63	57	50
3920	90	86	81	76	70	65	61	56	49

Percentage of cell viability is recorded in triplicates.

Table 3.5 Toxic effects of *Aspergillus* and *Penicillium* extracts from porridge on human mononuclear cells over 24, 48 and 72hrs of exposure.

	% Cell viability								
	24hrs			48hrs			72hrs		
Extracts conc. µg/kg	**20 µl/ml**	**40 µl/ml**	**80 µl/ml**	**20 µl/ml**	**40 µl/ml**	**80 µl/ml**	**20 µl/ml**	**40 µl/ml**	**80 µl/ml**
A. flavus									
1618	90	86	83	78	74	71	68	64	59
2532	85	80	76	71	66	60	56	52	47
2665	86	81	77	71	65	59	55	51	45
6403	84	79	74	68	63	56	51	45	40
10205	82	76	70	65	59	52	47	41	35
A. parasiticus									
3481	83	79	74	68	63	58	54	48	43
3659	82	77	72	66	61	56	52	46	40
5374	80	75	70	64	60	55	49	43	36
6930	80	74	69	63	58	51	46	42	36
9886	80	73	67	62	56	49	43	38	32
A. carbonarius									
17	96	92	88	83	79	76	72	69	65
A. niger									
0.3	99	96	94	91	91	90	89	87	85
20	98	94	90	85	81	77	73	70	67
31	97	95	91	87	82	79	76	70	65
50	95	90	86	82	77	72	68	63	59
A. ochraceus									
6	98	95	92	90	85	81	78	74	69
P. verrucosum									
60	94	90	86	82	77	74	70	65	61

Percentage of cell viability is recorded in triplicates.

Table 3.6 Toxic effects of *Aspergillus* and *Penicillium* extracts from water on human mononuclear cells over 24, 48 and 72hrs of exposure.

	% Cell viability								
	24hrs			48hrs			72hrs		
Extracts conc. µg/kg	**20 µl/ml**	**40 µl/ml**	**80 µl/ml**	**20 µl/ml**	**40 µl/ml**	**80 µl/ml**	**20 µl/ml**	**40 µl/ml**	**80 µl/ml**
A. flavus									
276	96	92	88	83	79	76	71	66	62
1292	90	85	81	76	71	65	58	53	49
1792	87	83	78	74	69	65	60	56	50
2570	84	79	75	69	64	60	55	51	45
5257	80	74	69	65	59	52	47	42	36
A. parasiticus									
2717	83	79	74	70	65	61	56	50	45
3727	82	77	72	66	61	55	49	45	40
5524	81	75	70	66	60	59	54	48	39
8048	79	73	66	61	54	48	43	39	33
16325	78	72	67	60	55	49	44	37	31
A. carbonarius									
-	-	-	-	-	-	-	-	-	-
A. niger									
10	95	91	88	84	80	81	77	72	67
347	94	90	85	84	78	72	67	64	60
747	90	85	80	76	70	66	62	57	53
1738	88	82	77	73	68	62	57	52	46
5000	81	76	70	63	55	50	45	39	33
A. ochraceus									
-	-	-	-	-	-	-	-	-	-
P. verrucosum									
8	96	91	87	84	80	76	73	70	66

Percentage of cell viability is recorded in triplicates.