Unraveling the potential of selected South African indigenous plants as biocontrol agents against aflatoxin-producing *Aspergillus* species

by

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DECLARATION

I, Sipho Hayley Chauke declare that the dissertation, which I hereby submit for the degree of Magistrae scientiae at the University of Pretoria, is my own work and has not previously been submitted for a degree at this or any other tertiary institution

……

Sipho Hayley Chauke

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ABSTRACT

Aspergillus flavus and *A. parasiticus* are saprophytic storage fungi, which primarily invade maize (*Zea mays*) and other cereal and legume crops. Contamination of stored grains with these fungal species reduces the quality of food and feed, which leads to yield loss. Moreover, *A. flavus* and *A. parasiticus* present a serious threat to human and animal health due to the production of aflatoxin B_1 (AFB_1), which is carcinogenic and is associated with stunting among children. Current control methods may be effective; however, due to the adverse residual effects associated with the use of synthetic chemical fungicides, alternate methods of pest control such as biodegradable agents are being researched. Plant extracts have been tested to be less toxic and environmentally friendlier compared to synthetic chemical fungicides. The goal of this study was to evaluate three plants, namely, *Erythrophleum lasianthum*, *Heteropyxis natalensis* and *Warburgia salutaris* for their antifungal activities and as seed treatments against known AFB_1-producing *A. flavus* and *A. parasiticus* isolates *in vitro*.

The extracts were screened using the broth microdilution method to obtain the minimum inhibitory concentration (MIC) values, of which the acetone, ethanol and water extracts of *W. salutaris* (0.117 mg/mL), *E. lasianthum* (0.234 mg/mL) and *H. natalensis* (0.469 mg/mL), respectively, showed noteworthy antifungal activity (≤ 1 mg/mL) against *A. flavus*. In contrast, the acetone extract of *W. salutaris* and the ethanol and water extracts of *H. natalensis* showed noteworthy antifungal activity (≤ 1 mg/mL) against *A. parasiticus* with MIC values of 0.117, 0.938 and 0.469 mg/mL, respectively. Following the antifungal screening, *E. lasianthum* (ethanol), *H. natalensis* (water) and *W. salutaris* (acetone) extracts were evaluated for their potential as AFB_1 reducers at 15, 30 and 75 mg/mL extract concentrations. Using spectrophotometry, the extract which reduced AFB_1 at 75 mg/mL the best was that made from the acetone extract of *W. salutaris* when compared to all the other tested extracts. *Warburgia salutaris* (75 mg/mL) was able to reduce AFB_1 by 49.27 % in liquid media while AFB_1 was least reduced by *E. lasianthum* (at 15 mg/ml), which was only able to reduce AFB_1 production by 12.82 %.

Furthermore, the selected five extracts, *E. lasianthum* (acetone), *H. natalensis* (acetone, ethanol and water) and *W. salutaris* (acetone) which showed good antifungal activity (against *A. flavus* and *A. parasiticus*) were screened for their potential toxicity on HeLa and HepG2 cell lines. All the extracts showed moderate to no cytotoxicity (> 50 µg/mL) on both cell lines with the exception of *H. natalensis* (water) and *W. salutaris* (acetone) extracts, which were toxic (≤ 50 µg/mL) to the HepG2 cell line, at the highest tested concentration (400 µg/mL).

Bioassay-guided fractionation of the ethanolic extract of *H. natalensis* indicated that the liquid-liquid partition butanol fraction was the most active against *A. flavus* and *A. parasiticus*. The butanol liquid-liquid partitioned fraction was further subjected to isolation and identification using Column chromatography and GC-MS, revealing five major compounds as potential fungal growth inhibitors present in the butanol fraction of *H. natalensis*.

Artificially inoculated maize seeds with *A. flavus* were treated with the three plant extracts, *E. lasianthum* (ethanol), *H. natalensis* (water) and *W. salutaris* (acetone), at 50 and 100 mg/mL and evaluated for their potential as seed treatments. The treated seeds were plated onto Potato Dextrose Agar (PDA) prior to storage, and three and six months after treatment. The extracts had no observable negative effects on seed germination. The extracts showed no significant fungal growth inhibition on treated maize seeds plated out prior to storage. However, after three months of storage, *H. natalensis* (water) and *W. salutaris* (acetone) at 100 mg/mL showed significant growth inhibition on maize treated seeds with fungal growth inhibition percentages of 65.00 and 70.50 %, respectively. After six months in storage, *E. lasianthum* (100 mg/mL) and *H. natalensis* (50 and 100 mg/mL) exhibited intermediate fungal growth inhibition of 48, 47 and 55 %, respectively. Therefore, these plants indicated the potential to be used as biological control agents against fungal contaminants of grains such as *A. flavus* in storage.

LIST OF RESEARCH OUTPUTS

Presentations at conferences:

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TABLE OF CONTENTS

LIST OF TABLES AND FIGURES

TABLES

FIGURES

ABBREVIATIONS

A	Acetone
ANOVA	Analysis of Variance
AFB_1	Aflatoxin B_1
AFB_2	Aflatoxin B_2
AFG_1	Aflatoxin G_1
AFG_2	Aflatoxin G_2
ARC	Agricultural Research Council
CAF	Central Analytical Facility
CAST	Council for Agricultural Science and Technology
CFU	Colony forming units
CYP	Cytochrome
DAFF	Department of Agriculture, Forestry and Fisheries
DMSO	Dimethyl sulphoxide
DMEM	Dulbecco's modified eagle medium
E	Ethanol
EDTA	Ethylene diamine tetra-acetic acid
ELISA	Enzyme-linked immunoassay
FDA	Food and Drug Administration
FAOSTAT	Food and Agriculture Organization Corporate Statistical Database
GC-MS	Gas chromatography-Mass spectrometry

H	Water
HeLa	Human cervix epithelial carcinoma cells
HepG2	Malignant human hepatoma cells
HPLC	High performance liquid chromatography
H_2O_2	Hydrogen peroxide
IC_{50}	Fifty percent minimum inhibitory concentration
IGF	Insulin-like growth factor
INT	*p*-Iodonitrotetrazolium chloride
LC-MS/MS	Liquid chromatography tandem-Mass spectrometry
MEA	Malt Extract Agar
MEB	Malt Extract Broth
MEM	Minimum essential medium
MFC	Minimum fungicidal concentration
MIC	Minimum inhibitory concentration
MRC	Medical Research Council
NaHClO	Sodium hypochlorite
PDA	Potato Dextrose Agar
SdH_2O	Sterile distilled water
SAGL	South African Grain Laboratory
SANBI	South African National Biodiversity Institute
SI	Selectivity index

TLC	Thin layer chromatography
UV	Ultraviolet

CHAPTER 1
General introduction

1.1 Introduction and motivation of the study

The genus *Aspergillus* comprises of some of the most diverse fungi that cause mould infections (Chandra et al., 1985). *Aspergillus* species are generally not host specific and are therefore agricultural, human and animal health threats (Vagra et al., 2004). *Aspergillus* species such as *Aspergillus flavus*, *Aspergillus nomius* and *Aspergillus parasiticus* produce toxic secondary metabolites known as aflatoxins (Gourama and Bullerman, 1995; Ehrlich et al., 2007; Klich, 2007a). There are six commonly known groups of aflatoxins namely aflatoxin B_1, B_2, G_1, G_2, M_1 and M_2 (Allcroft and Caunagham, 1963; Galvano et al., 2001; Klich, 2007a). One of the most potent aflatoxins produced by some of these fungi is aflatoxin B_1 (AFB_1), which is extremely toxic, causing aflatoxicosis in humans and animals (Probst et al., 2010). It is also carcinogenic and hepatotoxic in humans and animals (Nogueira et al., 2009; Pitt, 2000). The ingestion of aflatoxin-contaminated foods causing hepatocellular carcinomas has been reported in the rural areas of Mozambique and in the Eastern Cape Province of South Africa (Van Rensburg et al., 1974). An aflatoxicosis outbreak occurred in 1974 in west India where the ingestion of aflatoxin-contaminated grains caused hepatitis (Krishnamachari et al., 1975). Various other outbreaks of acute aflatoxicosis have been reported in Kenya, India and Thailand (Krishnamachari et al., 1975; Siriacha et al., 1990; Lewis et al., 2005). In 2004, there was an outbreak of acute aflatoxicosis in the eastern and central provinces of Kenya due to the ingestion of aflatoxin-contaminated maize (*Zea mays*), which also caused acute hepatotoxicity and death (Lewis et al., 2005).

Aspergillus flavus and *A. parasiticus* are typically considered storage fungi, which are mainly pathogenic to grain crops such as maize, groundnuts (*Arachis hypogea*), sorghum (*Sorghum bicolor*) and rice (*Oryza sativa*) (Klich, 2007b; Da Silva et al., 2012). The contamination of grains and crops with *A. flavus*, *A. parasiticus* and aflatoxins can

occur during crop development in the field (especially during drought stress conditions when soil temperatures are high), throughout to the storage of the grains (Cotty et al., 1994; Dohlman, 2003). Some factors, which have been reported to facilitate the contamination of grains with *A. flavus*, *A. parasiticus* and aflatoxin production, includes humidity, high temperatures and high moisture contents (Gourama and Bullerman, 1995). According to Cole et al. (1984), temperatures above 29 °C and drought stress increase the colonization of grains by these fungal species, thus also increasing the production of aflatoxins in and/or on the grains. The contamination of foods and feed with *Aspergillus* spp. and aflatoxins reduces its quality, rendering it unfit for human or animal consumption thus resulting in yield loss, which has a negative impact on the economy worldwide (Klich, 2007b; Kumar et al., 2007).

Current control measures of *A. flavus*, *A. parasiticus* and aflatoxin contamination in grains include physical and chemical methods and the use of atoxigenic *A. flavus* strains as biological control agents (Bluma et al., 2008b; Passone et al., 2008). Some of the physical methods used to minimize the contamination of grains include the use of modified atmospheres (optimal blend of pure nitrogen and oxygen in a permeable package or high barrier), proper soil management techniques, cold storage, aeration and rapid drying or radiation treatments of grains (Bluma et al., 2008a; Passone et al., 2008; Abbas et al., 2009). Other physical methods include crop fertility management strategies such as reducing crop heat and moisture stress by supplemental irrigation and providing pre-harvested grains with adequate nutrition, which is especially high in nitrogen (Abbas et al., 2009). Nitrogen deficiency has been reported in a few studies as a major contributor to grain crops being more susceptible to fungal and aflatoxin contamination (Jones, 1979; Bruns and Abbas, 2005; Abbas et al., 2009).

Many studies have reported that control with synthetic chemicals such as benzoic acid and ferulic acid have reduced the production of aflatoxins by *A. flavus* (Bilgrami et al, 1980; Chipley and Uraih, 1980; Zaika & Buchanan, 1987; Gourama and Bullerman, 1995). Other synthetic compounds include vanillic acid, chemical treatments with ammonia acids, food preservatives, pesticides and caffeic acids (Aziz et al., 1998; Jackson and Bullerman, 1999; Passone et al., 2008). Aliphatic acid-based

preservatives, propionic salts, and sorbic acid based commercial products are often used to prevent *A. flavus* and aflatoxin contamination of feed (Magan and Aldred, 2007).

Fungicides such as Bentex-T, Prochloraz, Tebuconazole, Thiabendazole, Carboxine and Fernfuran, have also been used to control *A. flavus* contamination of grains (Besri, 1992; Suberu, 2004; Mateo et al., 2017). Although these synthetic chemical fungicides may be effective, improper and excessive use is harmful to humans, animals and the environment (Gould, 1996; Zaker, 2016). Due to the increasing global awareness of the residual and carcinogenic effects of synthetic chemical fungicides, alternative products in the form of plant extracts and essential oils are being used to control plant fungal contamination and/or diseases (Gould, 1996; Lopez-Malo et al., 2005; Zaker, 2016).

In the last decade, several studies have reported the antifungal and antimicrobial activity of plant extracts and essential oils against fungi such as *A. flavus*, *Alternaria alternata*, *Aspergillus niger* and *Aspergillus fumigatus* among others (Sitara et al., 2008; Mesta et al., 2009; Nkomo and Kambizi, 2009; Reddy et al., 2010; Mahmoud et al., 2011; Gupta and Bhadauria, 2012; Cock and Van Vuuren, 2013; Gupta et al., 2014; Martins et al., 2014). This is due to the perception that plant extracts, plant constituents (natural plant products) and essential oils are less toxic, environmentally friendlier and biodegradable when compared to synthetic chemical fungicides (Salehan et al., 2013; Sahab et al., 2014).

Other studies, specifically targeting *A. flavus* and *A. parasiticus* growth and aflatoxin production have identified essential oils from plants such as clove (*Syzygium aromaticum*), pennyroyal (*Mentha pulegium*), eucalyptus (*Eucalyptus globulus*), cinnamon (*Cinnamomum verum*) and plant extracts from the *Polymnia sanchifolia* and *Agave* species to have fungal growth and aflatoxin reduction properties (Sinha et al., 1993; Pinto et al., 2001; Rasooli and Owlia, 2005; Sanchez et al., 2005; Bluma et al., 2008a).

The contamination of grains with *A. flavus*, *A. parasiticus* and aflatoxins has become a challenge, especially in developing countries where grains are the main source of food with very little grain inspection for *A. flavus*, *A. parasiticus* and aflatoxin contamination

being done (Sowley, 2016). In South Africa, about 25 to 33% of grain crops grown contribute to the total gross agricultural production, with maize being the most commonly cultivated (GrainSA, 2017). According to Chilaka et al. (2012) the contamination of maize grains with *Aspergillus* species and aflatoxins in South Africa were reported to be as high as 53 % and 149 µg/kg, respectively.

1.2 Problem statement

Fungal infections of maize crops in the field and during post-harvest storage damage the maize plants and grains by causing kernel decay (such as ear rot disease) through leaf and stalk diseases (Mouton, 2014). The main fungal contaminants of maize crops and grains are *Fusarium verticilliodes* and *Fusarium graminearum* (Ncube, 2008, Boutigny et al., 2011). However, *A. flavus* and *A. parasiticus* are also pathogens, which are the causal agents of ear rot disease in maize (Munkvold, 2003). In South Africa, 600 000 and more households depend on maize grains produced by subsistence farmers (Ncube et al., 2011). Therefore, the quality of the grain produced for consumption is important. Subsistence farming systems produce maize that often gets damaged before, during and post-harvest (Ncube et al., 2011). This increases the susceptibility of maize grains being contaminated with fungi especially during storage (Ncube et al., 2011). Furthermore, because most subsistence farmers use seeds from the previous harvest to plant new crops, this significantly contributes to the recurring systemic infection of grains with fungi and their mycotoxins (Ncube et al., 2011). Although, current control methods by synthetic fungicides minimize fungal contamination, synthetic fungicides are not always sustainable for the environment and affordable for subsistence farmers (South African Grain Laboratory, 2011). Therefore, there is a need for natural, biodegradable and affordable biological control agents against *A. flavus*, *A. parasiticus* and aflatoxin contamination on stored maize grains and crops in the field.

1.3 Aims and Objectives

The primary aim of this study was to investigate the reduction potential of *Erythrophleum lasianthum*, *Heteropyxis natalensis* and *Warburgia salutaris* against the growth of *A. flavus* and *A. parasiticus*, and their effects on the biosynthesis of aflatoxin B_1.

The specific objectives for this study were to:

- Investigate the antifungal activities (minimum inhibitory concentration) of acetone, ethanol and water extracts of *E. lasianthum*, *H. natalensis* and *W. salutaris* against *A. flavus* and *A. parasiticus*.
- Investigate the effect of the selected plant extracts on the production of AFB_1 by *A. flavus*.
- Investigate the cytotoxicity of the extracts on stomach and liver (substitute) cell lines.
- Isolate the active compound(s) from the plant(s) showing antifungal activity against *A. flavus* and *A. parasiticus* growth.
- Investigate the effect of the selected plant extracts as seed treatments *in vitro*.
- Evaluate the shelf-life of the selected plant extracts as seed treatments over a period of six months.

1.4 Dissertation structure

The structure of this dissertation is prepared as described below:

Chapter 1: Background information, motivation for the study, the problem statement and the aims and objectives for this study is provided.

Chapter 2: This chapter provides a review of relevant literature relating to aflatoxigenic *Aspergillus* species and the production of aflatoxins with emphasis on aflatoxin B_1. This chapter also briefly evaluates current control methods, the use of plant-derived products as antifungal agents, and their use as seed

treatments. A succinct description and relevant background on the plants selected for the study is also given in this chapter.

Chapter 3: In this chapter the antifungal and aflatoxin reduction potential of the selected plants is evaluated. Furthermore, the plant extracts with the best antifungal activity are evaluated for their toxicity on stomach and liver cell lines.

Chapter 4: This chapter includes the isolation of potential active compounds from the *H. natalensis* plant extract, which may be effective against the growth of *Aspergillus* species and the production of AFB_1.

Chapter 5: This chapter evaluates the efficacy and shelf-life of the selected plants as maize seed treatments *in vitro*.

Chapter 6: A general discussion of the study and suggestions for future research are given in this chapter.

Chapter 7: A compilation of all the literature used as references for this study is provided.

CHAPTER 2
Literature review

2.1 *Aspergillus* species

2.1.1 Taxonomy, morphology and distribution of *Aspergillus* spp.

Aspergillus species are classified as Ascomycetes, which is a division in which fungal species with and without teleomorphic stages (teleomorph- reproductive form of a fungus) are grouped into (Scheidegger and Payne, 2003). In the past, *Aspergillus* species were classified as Deuteromycetes, however, morphological, physiological and biochemical-based studies indicated otherwise (Ascomycetes) (Alexopoulos et al., 1996). The *Aspergillus* genus was initially divided into 18 groups, after which morphological and molecular phylogenetic data, led to its division into six subgenera with 18 sections (Gams et al., 1985; Raper and Fennell, 1965; Scheidegger and Payne, 2003). The initial six subgenera were *Aspergillus*, *Circumdati*, *Clavati*, *Fumigati*, *Ornate* and *Nidulantes* (Tamura et al., 2000; Scheidegger and Payne, 2003). However, this grouping was later disputed due to phylogenetic data that indicated that only *Aspergillus*, *Fumigatus* and *Nidulantes* are part of a monophyletic taxonomy in terms of *Aspergillus* species (Peterson, 2000).

Aspergillus taxonomy has been described as very complex because some species within this genus cannot be distinguished using morphological parameters, such as colour and conidiophore texture, only (Frisvad et al., 2005; Pildain et al., 2008). Furthermore, the *Aspergillus* genus, which is divided into six subgenera, is also divided into sections (Gugnani, 2003). This study will mainly focus on *Aspergillus* section *Flavi*, which consists of nine known species namely: *A. flavus*, *A. parasiticus, A. oryzae*, *A. zonatus*, *A. clavato-flavus*, *A. tamarii*, *A. flavo-furcatis*, *A. subolivaceus*, *A. avenaceus* and two varieties, which were identified as *A. flavus* var. *columnaris* and *A. oryzae* var. *effucus* (Raper and Fennell, 1965). Among, these known section *Flavi* species, *A. flavus* and *A. parasiticus* are classified as the main aflatoxin-producing and

stored product-spoiling fungi amongst other species in the *Aspergillus* genus (Kumeda and Asao, 2001; Rigo et al., 2002).

In addition, *Aspergillus flavus* isolates are grouped into either the L or S strain sclerotial morphotypes (Saito and Tsuruta, 1993). The L strain morphotype is associated with the production of abundant conidiospores and sclerotia greater than 400 µm in diameter (Cotty, 1989; Horn and Dorner, 1999). In contrast, the S strain morphotype, also referred to as *A. flavus* var. *parvisclerotigens*, is associated with the production of fewer or less conidiospores and numerous sclerotia smaller than 400 µm in diameter (Cotty, 1989; Horn and Dorner, 1999). The two morphotypes can also be differentiated in terms of their ability/potential to produce aflatoxins. The S morphotype typically produces greater amounts of aflatoxins when compared to the L morphotype, which typically produces minute to no aflatoxins at all (Bayman and Cotty, 1993; Horn and Dorner, 1999; Tran-Dinh et al., 1999; Novas and Cabral, 2002; Vaamonde et al., 2003; Mphande et al., 2004; Pildain et al., 2004; Chang et al., 2006). The following sections will focus on both *A. flavus* and *A. parasiticus*, but with emphasis placed on *A. flavus*.

Aspergillus species are morphologically different from other fungal species by the colour and texture of their conidiophores and conidia (Gourama and Bullerman, 1995; Krishnan et al., 2009; Vagra et al., 2011). *Aspergillus* species are characterized mainly by a distinctive spore-bearing structure known as an aspergillum (Gourama and Bullerman, 1995). These fungal species produce elongated flask-shaped projections, which arise from vesicles known as phialides (Pitt and Hockings, 1985; Klich, 2007a). *Aspergillus flavus* and *A. parasiticus* have conidial heads, conidia, conidiophores (stalk-like structures rising upwards from the foot cells) and sclerotia (hard and compacted clusters of mycelia which vary in shape and size) (Shearer et al., 1992; Gourama and Bullerman, 1995, Krishnan et al., 2009). The conidiophores of *A. flavus* and *A. parasiticus* are divided into stipes, foot cells and the vesicles (Gourama and Bullerman, 1995). The conidiophores have swollen apexes (vesicles), which are elongated when the fungal cultures are still young, however, as the culture ages; the vesicles become bulbous (Gourama and Bullerman, 1995). This is however influenced by the composition of the substrate on which the fungi grow on, which in turn influences

the shape and the diameter of the vesicles (Raper and Fennell, 1965; Gourama and Bullerman, 1995). On the conidial heads of *A. flavus* and *A. parasiticus*, the phialides produce conidia also commonly known as spores (Gourama and Bullerman, 1995; Klich, 2002). The colour of the conidia produced makes it easier to differentiate *A. flavus* from *A. parasiticus* because *A. flavus* conidia are light yellow to green in colour whereas *A. parasiticus* conidia are more of a dark to olive green colour (Figure 2.1) (Gourama and Bullerman, 1995; Klich, 2002).

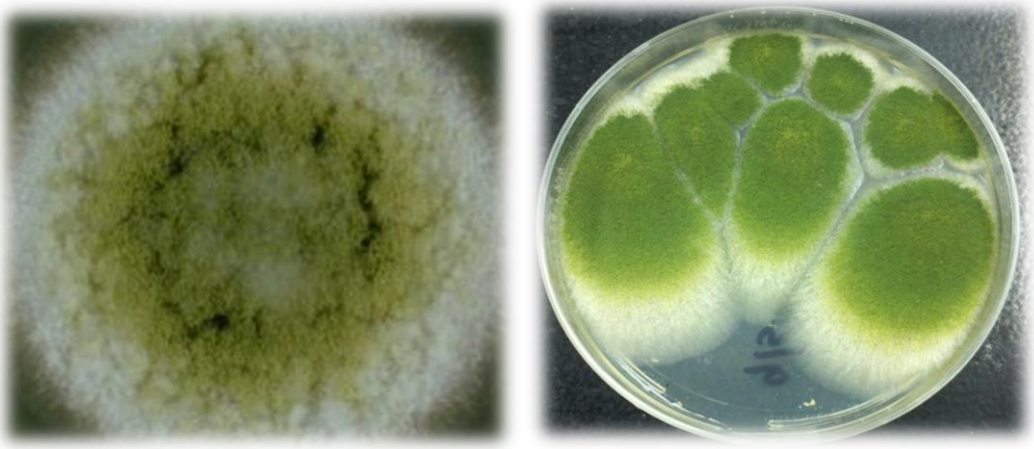


Figure 2.1: *Aspergillus flavus* (left) and *A. parasiticus* (right) (Microatlas, 2011; Aglifesciences, 2014).

Amongst, some of the differences between the two *Aspergillus* species, the distribution of the two also varies. The distribution of *A. flavus* has been reported to occur in all the major biomes in all the different climatic zones (Manabe and Tsuruta, 1978; Klich, 2002; Klich, 2007). It has however also been frequently isolated from temperate zones with warmer temperatures at latitudes ranging between 26 and 35 °C (Manabe and Tsuruta, 1978; Klich, 2002). The contamination of field crops with *Aspergillus* species and aflatoxins is often associated with drought stressed crops growing in temperate zones, which experience high temperatures (CAST, 2003). The production of conidia by *Aspergillus* species is accountable for the distribution of *A. flavus* and *A. parasiticus* worldwide due to the conidia being easily dispersible by wind and insects (Hedayati et al., 2007).

Within the *Aspergillus* genus, aflatoxigenic fungi occur at different geographical locations even though most are soil microorganisms (Diener and Davis, 1965; Dorner et

al., 1989). *Aspergillus parasiticus* occurs in more tropical and/or subtropical regions, where the environment is warmer (Diener and Davis, 1965). Its spores are often found more in the soil than in the air when compared to *A. flavus*, which occurs in warm temperate zones and whose spores occur more in the air than in the soil (Diener and Davis, 1965). Therefore, *A. flavus* contamination is more prevalent in maize (*Zea mays*) while *A. parasiticus* contamination is more prevalent in groundnuts or peanuts (*Arachis hypogaea*) (Gourama and Bullerman, 1995).

2.1.2 Infection of plants and stored products by *Aspergillus* spp.

Aspergillus flavus and *A. parasiticus* are saprophytic fungi, which survive and out-compete other microorganisms for substrates either on plants or in the soil (Bhatnagar et al., 2000; Scheidegger and Payne, 2003). This is due to their ability to produce sclerotia, which are able to germinate and produce hyphae or conidia, which can be dispersed into the air and onto the soil and plants (Hedayati et al., 2007).

According to Scheidegger and Payne (2003), the presence of plant and animal debris in the field promotes fungal (*A. flavus* and *A. parasiticus*) growth in the field. The possible mechanism by which crops are infected with *Aspergillus* species is shown in Figure 2.2 (Scheidegger and Payne, 2003). Dormant sclerotia present in the soil and on plant and animal debris can spread through abiotic factors such as wind or by other biotic factors such as insects (Scheidegger and Payne, 2003). The sclerotia from the surrounding plant and animal debris can act as the primary inoculum that can infect and colonize the crops in the field. When environmental conditions are hot and dry leading to drought stress, the sclerotia are able to germinate and produce more sclerotia or mycelia on the crops and on maize kernels. The sclerotia are able to enter and spread through the maize silk (tissue) thus spreading towards the kernels. The fungus may also go as far as invading the cell walls via the air spaces present at the junction between the bracts and the rachis (cob) of the maize plant (Smart et al., 1990; Payne, 1998).

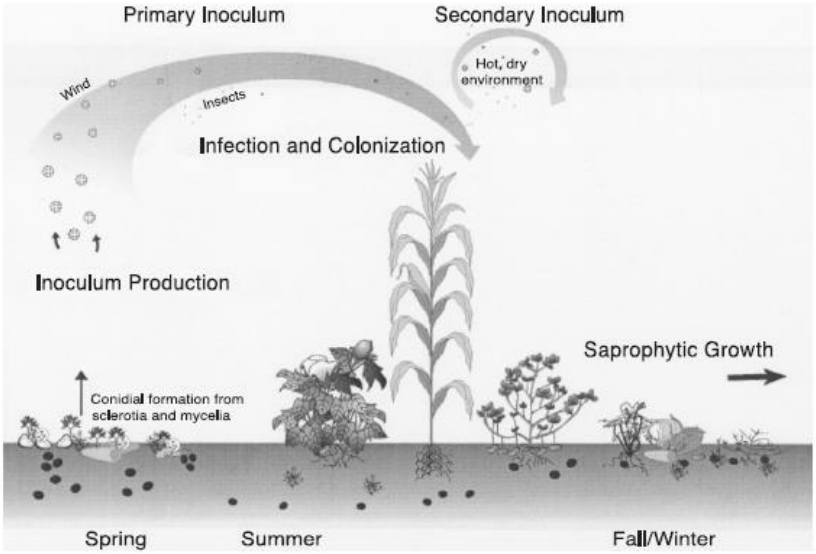


Figure 2.2: A diagram of crop infection with *Aspergillus flavus* through the spread of conidia by wind (Scheidegger and Payne, 2003).

Some of the factors that influence the contamination of crops with *Aspergillus* species include drought stress, high temperatures (≥ 25 °C), damage caused by insects and the type of soil and tillage used in the field (Diener et al., 1987; Lisker and Lillehoj, 1991; Zablotowicz et al., 2007).

Since cereal grains are one of the most important food sources in the world, especially in developing countries, they are vulnerable to fungal and mycotoxin contamination during storage (Ng'ang'a et al., 2016). In South Africa, most of the cereal grain (such as maize) production is done by the commercial farmers; however, some small-scale farmers also sell their produce commercially and/or retain some for later consumption (Ekwomadu et al., 2018). Therefore, the quality of the grains needs to be maintained and different farmers may preserve these grains differently (Thamaga-Chitja et al., 2004). Commercial farmers have access to the correct storage facilities (such as warehouses with optimal and controlled conditions) and drying technologies such as batch drying systems, fluidized bed dryers, infrared dryers and grain-rotary dryers (Muthukumarappan and Singha, 2016). In contrast, some small-scale farmers still use traditional storage techniques such as storing grains in sacks, woven baskets, silo structures made of twigs or clay, small bags with cow dung and/or ash, buckets,

hanging the cobs in the open air, and sun drying the grains by spreading them on the ground and exposing the grains to the sun and the wind/air (Motte et al., 1995; Addo et al., 2002;Thamaga-Chitja et al., 2004; Kankolongo et al., 2009; Wambugu et al., 2009; Muthukumarappan and Singha, 2016). Although some of these storage and drying methods are effective, improper grain storage due to lack of the technology and in some instances the knowledge of how to store the grains, may lead to poor management and storage of the grains, thus leading to the susceptibility of the grains to fungal and mycotoxin contamination in storage (Harein and Davis, 1992; Sauer et al., 1992; Bankole and Adebanjo, 2003). In most cases, grains are contaminated with fungi while in the field and rapidly develop and produce mycotoxins in suitable conditions during storage (Turner et al., 2002).

Grain contaminating *Aspergillus* species thrive in storage, especially if the temperature and moisture content of the storage facilities are suitable for growth (White, 1995). These two physical factors (temperature and moisture content) also contribute to the fungus being able to survive by producing spores and mycotoxins in/on the grains (White, 1995). *Aspergillus flavus* and *A. parasiticus* growth and aflatoxin production are especially problematic on stored grain mainly in hot and humid countries (Villers et al., 2014). During storage, a combination of heat and humidity build up and promote the exponential growth of the fungi, thus increasing the production of aflatoxins (Hell et al., 2010; Villers et al., 2014). Typically, fungal growth occurs optimally at 30 °C (25-43 °C), 85 % relative humidity (62- 99 %) in combination with the moisture of the kernel being 18 % (13-20 %), in storage (Villers et al., 2014). Aflatoxin production by *A. flavus* and *A. parasiticus* in storage is often inhibited when maize grains are stored at temperatures below 18 °C with the kernel moisture content below 13 % (Villers et al., 2014). The combination of these two factors also inhibits the growth of the fungi (Villers et al., 2014). Often, the presence of *Aspergillus* growth on maize grains/kernels does not occur uniformly from one kernel to the next and does not necessarily indicate the contamination of the grains with aflatoxins, since the production of aflatoxins only occurs optimally under certain conditions (27-30 °C temperature, 17-18 % moisture content and higher) (Robertson, 2005).

2.2 Aflatoxins

2.2.1 Aflatoxin chemistry

Aflatoxins are chemically difuranocoumarin derivatives produced by enzymes from the polyketide pathway present in some *Aspergillus* species (Klich, 2007a; Dzhavakhiya et al., 2016). The polyketide (synthase) pathway that produces aflatoxins also produces hydrophobic pigments, which constitute as fungal melanin (Pal et al., 2014). The main aflatoxins produced naturally are aflatoxins B_1, B_2, G_1, and G_2 (Figure 2.3) (Sweeney and Dobson, 1998). The nomenclature for these aflatoxins was based on the fluorescent colours, which the aflatoxins emit under ultraviolet light on Thin layer chromatography (TLC) plates (Wogan, 1966; Sweeney and Dobson, 1998). Aflatoxins B_1 (AFB_1) and B_2 (AFB_2) fluoresce a blue colour while aflatoxins G_1 (AFG_1) and G_2 (AFG_2) fluoresce a yellow-green colour (Wogan, 1966; Sweeney and Dobson, 1998). There are other aflatoxins, which exist due to metabolism of these main groups of aflatoxins. These are aflatoxins M_1 and M_2 (found in milk), which are hydroxylated metabolites from aflatoxins B_1 and B_2 (Sweeney and Dobson, 1998; Richard, 2007).

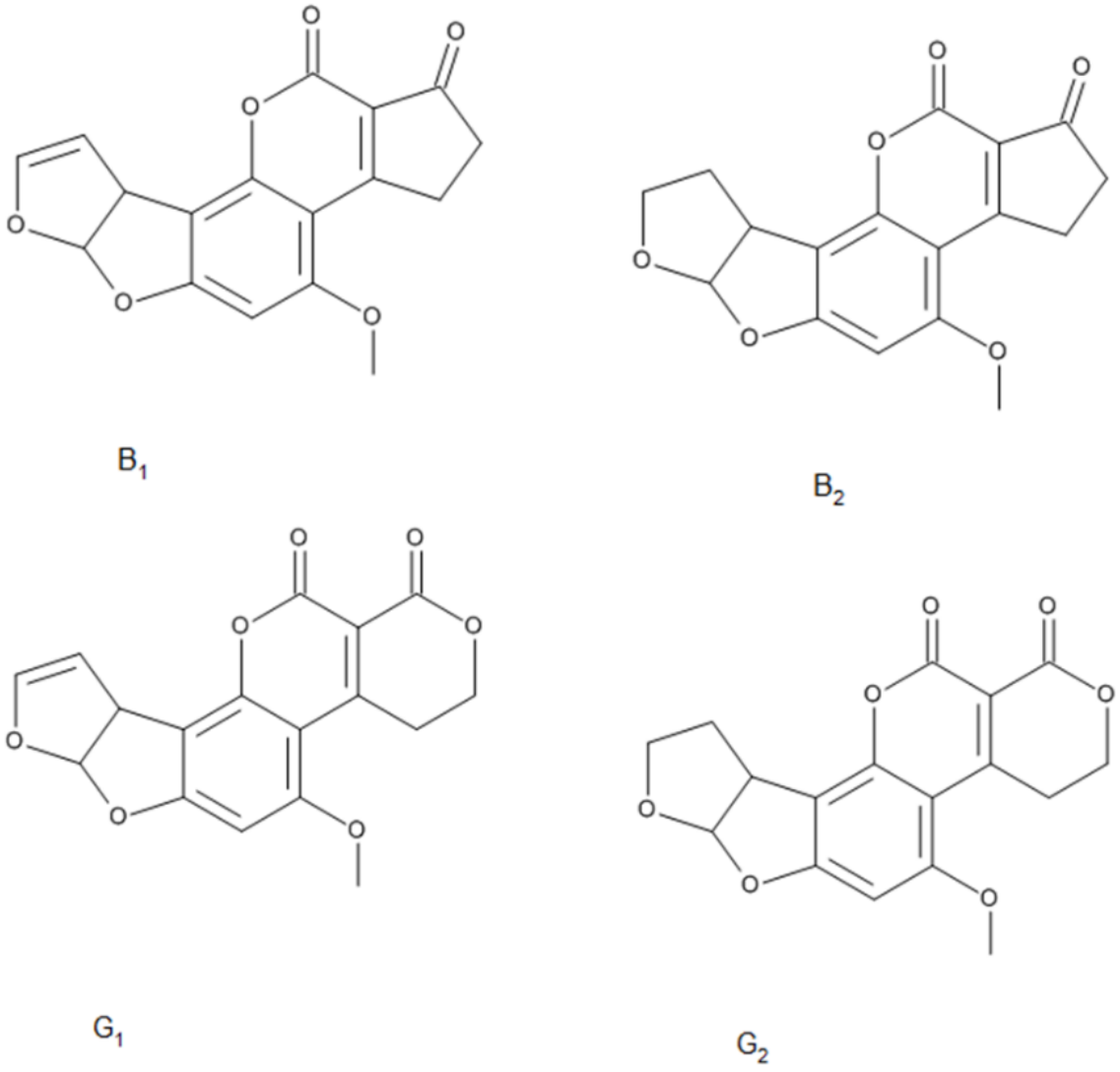


Figure 2.3: Chemical structures of aflatoxins B_1, B_2, G_1 and G_2.

The biosynthetic pathway of aflatoxins starts with the conversion of an acetate molecule and malonyl coA to a decaketide norsolorinic acid precursor using the enzyme polyketide synthase (Bhatnagar et al., 1994; Trail et al., 1995; Bennet et al.,1997; Minto and Townsend, 1997; Sweeney and Dobson, 1998). The first precursor then undergoes 5 to 12 enzymatic reactions to form AFB_1 and AFG_1 (Sweeney and Dobson, 1998). During these enzymatic reactions, intermediates such as averantin, averufanin, 1-hydroxyversicolorone, versiconal and versicolerin A are formed (Bhatnagar et al., 1994; Sweeney and Dobson, 1998; Gourama and Bullerman, 1995). The chemical structures of AFB_1 and AFG_1 have dihydrofuran rings produced from dimethyl sterigmocystin

(DMST), while AFB_2 and AFG_2 have tetrahydrobisfuran rings produced from dihydromethylsterigmatocystine (DHMST) (Figure 2.3) (Sweeney and Dobson, 1998).

One of the earlier studies on aflatoxins reported that the molecular formulas for each of the main naturally produced aflatoxins were $C_{17}\ H_{12}\ O_6$ (B_1), $C_{17}\ H_{14}\ O_6$ (B_2), $C_{17}\ H_{12}\ O_7$ (G_1) and $C_{17}\ H_{14}\ O_7$ (G_2) (Hartley et al., 1963). The study went on to establish the molecular weights, fluorescence emission and melting points for each of the main aflatoxins as indicated in Table 2.1.

Table 2.1: The chemical properties of the four main groups of aflatoxins (Hartley et al., 1963; van Dorp et al., 1963; Wogan, 1966).

Aflatoxin	Molecular formula	Molecular weight	Melting point (°C)	Fluorescence emission (mμ)
B_1	$C_{17}\ H_{12}\ O_6$	312	268-269	425
B_2	$C_{17}\ H_{14}\ O_6$	314	286-289	425
G_1	$C_{17}\ H_{12}\ O_7$	328	244-246	450
G_2	$C_{17}\ H_{14}\ O_7$	330	237-240	450

2.2.2 Toxicity

Generally, mycotoxins have adverse effects on plants, microorganisms, animals and humans (Bullerman, 2003). However, the toxicity of these toxins depends on the dosage/dose of the mycotoxins (Bullerman, 2003). Among, all the known mycotoxins, aflatoxins are the most potent and carcinogenic mycotoxins mainly targeting the liver in all animals including humans (Eaton and Groopman, 1994; Bullerman, 2003). Diseases which manifest due to the consumption of aflatoxins are called aflatoxicoses (Bennett and Klich, 2003). The susceptibility of humans and animals to aflatoxin toxicity may be influenced by a number of factors namely: differences in age, weight, diet, sex and exposure to other mycotoxins and infectious agents (Bennett and Klich, 2003).

In humans and animals, exposure to aflatoxins usually leads to one of three consequences (Bennett and Klich, 2003). The first consequence is exposure to a large aflatoxin dose, which can lead to acute illness due to liver cirrhosis, which eventually results in death (Bennett and Klich, 2003). The second consequence is exposure to chronic sub-lethal doses of aflatoxins, which result in immunological and nutritional consequences (Gupta, 2011). The third consequence is exposure to any dose of aflatoxins repeatedly, which results in a cumulative effect thus increasing the risk of the affected individual getting cancer (Howard et al., 1990; Williams et al., 2004).

Aflatoxins have also been reported to have deleterious effects on the reproductive and developmental systems by affecting human (mainly children) growth and the balance of the hormones while also affecting the growth of the fetus and gestation in pregnant individuals (Kourousekos and Lymberopoulos, 2007). In developing countries, ingestion of aflatoxin-contaminated foods, is more common in humans and agricultural animals (such as cattle) compared to developed countries where domestic animals (such as dogs) are affected more (Klich, 2009). The ingestion of aflatoxins has also been associated with childhood impaired growth, otherwise known as childhood stunting (Gong et al., 2016). This is because many weaning products in developing countries are typically made from maize and groundnuts, therefore making weaning foods more susceptible to aflatoxin contamination (Egal et al., 2005).

The level of aflatoxin exposure of children to adults is higher because of the body weight of children, which makes the impact of aflatoxins on growth greater in children than in adults (Gong et al., 2016). The possible mechanism of this was suggested to be due to the disruption of the pathway with insulin-like growth factors (IGF) by the aflatoxin, through liver toxicity (Gong et al., 2016). However, other possible mechanisms have been stated to be due to the aflatoxins' ability to have an immunosuppressive effect which consequently increases susceptibility to infections and nutritional impairment through suppressing appetite and reducing the absorption of nutrients (Egal et al., 2005). Another possible mechanism was postulated to be due to aflatoxins promoting intestinal damage by inhibiting protein synthesis, which results in the

absorption of essential nutrients being minimal therefore impairing subsequent growth (Smith et al., 2012).

2.2.3 Metabolism

When AFB_1 is ingested, its biotransformation or metabolism takes place in the liver, specifically in the microsomes (subcellular fraction of the liver containing many drug-metabolizing enzymes) (Gallagher et al., 1996; Vondracek et al., 2001; Wild and Turner, 2002). In humans, the biotransformation of AFB_1 through catabolism is mediated by Cytochrome P_{450} monooxygenase enzymes, namely: CYP1A2 and CYP3A4, which oxidize the transfer and separation of electrons from the biotransformation reactions (Figure 2.4) (Vondracek et al., 2001; Wild and Turner, 2002; Bbosa et al., 2013). The enzyme CYP1A2 converts AFB_1 from an exoepoxide (toxic, mutagenic and carcinogenic) to an endoepoxide (toxic only) molecule, while the CYP3A4 enzyme converts AFB_1 to AFB_1-exo-8,9-epoxide and aflatoxin Q_1, which is excreted in urine (Wild and Turner, 2002; Turner, 2013). The biotransformation products that are not excreted (AFB_1-exo-8, 9-epoxide) are conjugated to dialdehydes, which form Schiff bases (subclass of imines with the general compound structure being $R_2C=NR$) and dialcohols, which are excreted in urine (Hayes et al., 1993; Knight et al., 1999; Wild and Turner, 2002). The dialdehydes form conjugates with blood proteins such as albumin, which remain in the blood stream as aflatoxin-protein adducts of which very little of these aflatoxins get excreted into the urine (Nassar et al., 1982; Wacoo et al., 2014). Hepatotoxicity then occurs due to the accumulation of aflatoxin-protein adducts leftover from the purification of blood by the liver (Nassar et al., 1982; Wacoo et al., 2014).

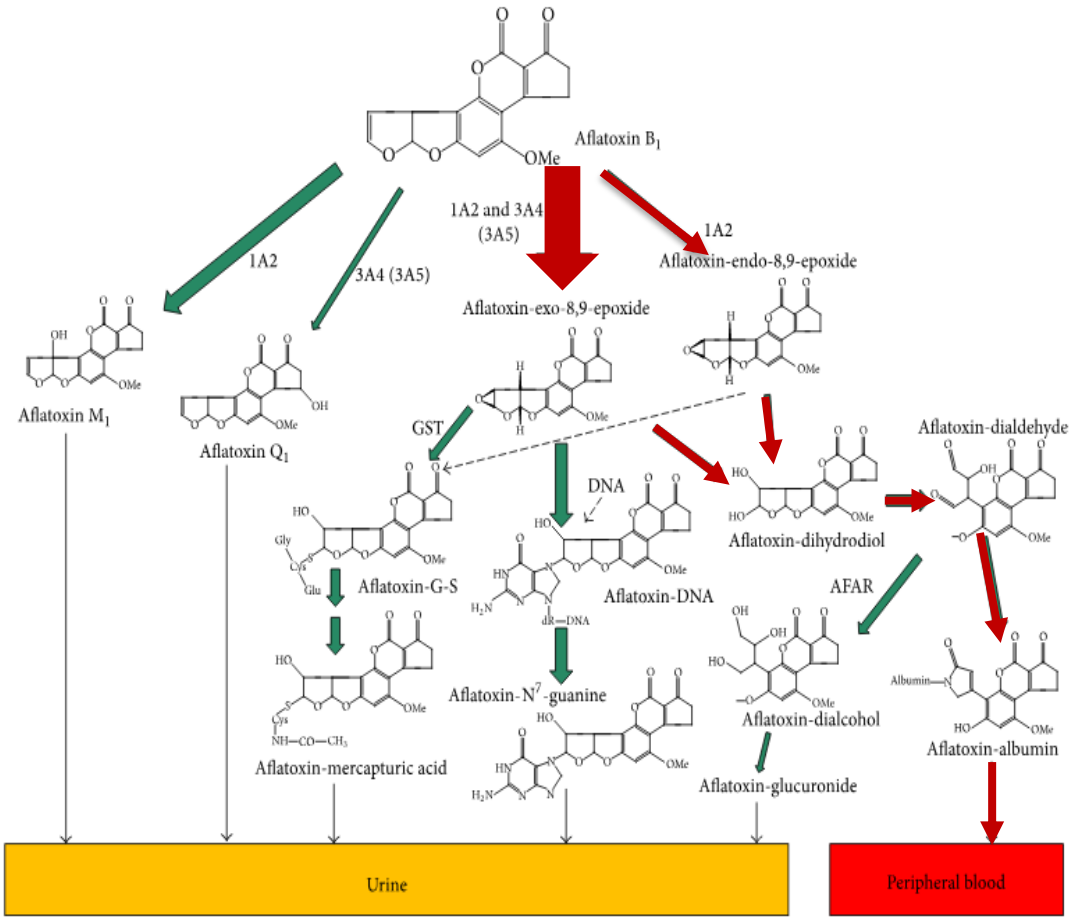


Figure 2.4: Aflatoxin metabolism using the Cytochrome P_{450} monooxygenase enzymes, with emphasis on aflatoxin conjugates which enter the blood stream (Wild and Turner, 2002).

2.2.4 Conditions for aflatoxin production

The production of aflatoxins has been described as a process that is a consequence of the combined effects of the environment and the substrate on which the fungus is growing on (Gourama and Bullerman, 1995). Factors that influence the production of aflatoxins include physical factors such as temperature, relative humidity and pH and nutritional factors such as the type of substrate on which the fungus is growing (Gourama and Bullerman, 1995). The substrate forms the basis onto which other factors such as temperature and relative humidity can influence the production of aflatoxins (Gourama and Bullerman, 1995).

2.2.5 Aflatoxin contamination

Aflatoxin contamination of stored grains and crops could occur before harvesting, during crop maturation in the field (especially in crops experiencing drought stress and growing in high soil temperatures) and during improper storage of the grains (Dohlam, 2003). For example, the contamination of groundnuts with aflatoxins can occur before, during and post-harvest (in storage) on the groundnut pods and seeds (Guchi, 2015).

Various studies have reported the optimum temperature for the production of aflatoxins by *A. flavus* and *A. parasiticus* in liquid media and on cultured maize to be 25 °C and 25-35 °C, respectively (Diener and Davis, 1966, Durakovic et al., 1987; Gqaleni et al., 1997; Klich, 2007b). In some studies, it was noted that no aflatoxins were produced at temperatures below 13 °C and at temperatures above 42 °C (Diener and Davis, 1966; Gourama and Bullerman, 1995). The production of aflatoxins is said to be at its maximum after a 15-day incubation period at 20 °C or after an 11-day incubation period at 30 °C (Diener and Davis, 1966). Therefore, as the temperature increased, a decrease in the incubation period required by the fungus was noted (Jarvis, 1971). Jarvis (1971) indicated that the production of aflatoxins is higher when the flasks in which the fungi are growing in, are shaken when compared to those growing in stationary flasks. Therefore, the production of aflatoxins in cereal grains due to the presence of carbon sources such as glucose, fructose and sucrose, act as natural substrates for aflatoxigenic fungi to produce aflatoxins (Jarvis, 1971; Gourama and Bullerman, 1995). Furthermore, the presence of carbon sources in cereal grains such as maize, rice (*Oryza sativa*) and peanuts/groundnuts, makes these grains more susceptible to carbon source utilization by *A. flavus* and *A. parasiticus* (Mellon et al., 2000; 2005; Klich, 2007b).

Many factors affecting aflatoxin production by *A. flavus* include the climate of the region, the type of soil, the daily temperatures and net evaporation (Ono et al., 1990; Brown et al., 2001; Bankole and Mabekoje, 2004; Fandohan et al., 2005). In storage, the production of aflatoxins is promoted mainly by the humidity, the temperature and the aeration in storage (Abrar et al., 2012). The production of aflatoxins by *A. flavus* occurs at temperatures between 12 and 34 °C, in storage (Brackett, 1989). However, it has

been reported that aflatoxin production is halted at temperatures greater than 36 °C (Brackett, 1989). Therefore, optimum aflatoxin production in storage occurs at temperatures between 28 and 30 °C (Brackett, 1989).

2.2.6 Methods used for the detection of aflatoxins

When the contamination of foods and feed with aflatoxins became apparent worldwide, methods for extracting, detecting and quantifying aflatoxins were developed (Pons and Goldblatt, 1969). The developed detection methods were categorized or grouped into chromatographic, immunochemical and spectroscopic detection methods (Wacoo et al., 2014). One of the chromatographic methods, which became the standard technique for aflatoxin detection, was Thin layer chromatography (TLC) (Klich, 2007b). This method is still being used today due to its simplicity and affordability (Klich, 2007b). Some of the other popular methods for detecting aflatoxins include High-performance liquid chromatography (HPLC), fluorescence spectrometry, enzyme-linked immunosorbent assay (ELISA) and immunosensors (Wacoo et al., 2014).

The detection of aflatoxins in food and feed usually requires sample preparation, where the aflatoxins are extracted from the food samples by using polar organic solvents such as methanol and chloroform mixed with water (Wacoo et al., 2014). This is due to the ability of the aflatoxins to be soluble in polar organic solvents (Bertuzz et al., 2012; Wacoo et al., 2014). The extraction of aflatoxins during sample preparation is often followed by a clean-up step, which incorporates immunoaffinity column chromatography for aflatoxin purification and concentration just before quantifying the aflatoxins using chromatography methods such as HPLC (Ma et al., 2013).

2.2.7 Thin layer chromatography

Thin layer chromatography is one of the most widely used methods for aflatoxin analysis (De Iongh et al., 1964; Wacoo et al., 2014). The general principle for this method, in terms of aflatoxin detection, is that a mobile phase, which usually comprises of solvents such as acetronitrile, methanol and water, carries the sample along the stationary phase (silica gel) (Betina, 1985; Wacoo et al., 2014). The differences in the solubility of the analytes of the stationary and mobile phases determine the distribution

of the aflatoxins on the TLC plate (Wacoo et al., 2014). The main reason behind the TLC method being a quick and effective method for sample separation is due to its ability of the different components present in the sample being able to adhere to the stationary phase more than the mobile phase or vice versa (Wacoo et al., 2014). Although the TLC method is an effective way for the separation of components present in samples, it is prone to experimental errors such as lack of precision during plate development, application of the samples on the TLC plate and/or interpretation of the results obtained after plate development (Papp et al., 2002; Stroka and Anklam, 2002; Wacoo et al., 2014).

2.2.8 High-performance liquid chromatography (HPLC)

This is the most popular method for the separation and identification of organic compounds in samples especially aflatoxin-contaminated samples (Li et al., 2011; Wacoo et al., 2014). The general principle of using HPLC for aflatoxin separation and determination also involves a stationary and mobile phase flowing through a solid adsorbent (column) (Li et al., 2011). This method is based on the different affinities of the sample for the mobile and stationary phases, moving through the column (Wacoo et al., 2014). The HPLC method provides more accurate and quicker aflatoxin detection methods in a short space of time when compared to the TLC method (Herzallah, 2009; Wacoo et al., 2014). However, using this method for analyzing aflatoxins requires rigorous sample purification, which may be time consuming and tedious (Li et al., 2011; Wacoo et al., 2014). Therefore, this method is often used together with Mass spectrometry (MS) to make aflatoxin determination easier after sample separation (Takino and Tanaka, 2008). This is because MS is highly selective and sensitive during sample analysis, as it gives quantitative data that is reliable and that can be applied in routine analyses for multiple toxins at the same time in a single run (Liao et al., 2011). Mass spectrometry in combination with HPLC also enables unambiguous and more stringent conformation of the sample in terms of the quantity and qualitative presence of certain substances in the sample, when compared to Fluorescence detection (FLD) coupled with HPLC (Cavaliere et al., 2007).

2.3 Current control and prevention methods against growth of *Aspergillus* spp. and aflatoxin production

Generally, the contamination of foods and feed by mycotoxins is controlled by using synthetic chemical fungicides, physical methods and biological agents (Yousef and Marth, 1981; Bullerman et al., 1984; Bluma et al., 2008a; Abbas et al., 2009). Small-scale farmers in some developing countries rely mainly on sun-drying grains, in an attempt to avoid fungal and mycotoxin contamination (Hell et al., 2000). Grains are often dried on raised platforms to avoid contact with the soil; however, other small-scale farmers use bamboo thatch or wood covered containers and/or cover the grains on raised platforms with a mud or thatch sheet (Hell et al., 2000).

The following section will focus on the different modes of control, which are currently used to control the growth of *Aspergillus* species and the production of aflatoxins on foods and feed.

2.3.1 Physical control methods

Some of the physical methods which have been used to minimize *Aspergillus* species and aflatoxin contamination include the use of modified atmospheres (an optimal blend of pure nitrogen and oxygen in a permeable package), proper soil management strategies and crops naturally resistant to insects (Abbas et al., 2009, Navarro et al., 2012). Other methods include supplemental irrigation, which reduce grain crop heat and moisture stress, use of short season hybrid seeds or crops and incorporating early planting schedules (Abbas et al., 2009). These strategies are carried out to avoid drought stress, which makes grains and crops more susceptible to *Aspergillus* species infection and aflatoxin production in the grains and crops (Abbas et al., 2009). Nitrogen deficiency in grain crops has been reported to be one of the major contributors to grain crop susceptibility to *Aspergillus* species infection (Jones, 1979; Bruns and Abbas, 2005; Abbas et al., 2009). Therefore, to reduce nitrogen deficiency in grain crops, fertility management strategies such as providing adequate nutrition, especially high in nitrogen, reduces susceptibility of grain crops to fungal and mycotoxin contamination (Abbas et al., 2009). Physical methods commonly used for aflatoxin detoxification

include the treatment of foods and feed with UV light, pasteurization, cooking and microwaving the contaminated foods and/or feed (Mishra and Das, 2003). However, the above-mentioned methods are not very effective in detoxifying the toxins from the contaminated foods and feed (Mishra and Das, 2003). An example of one effective control method is the use of adsorbents such as clay, which are often used to remove aflatoxins from feed intended for animal consumption (Masimango et al., 1979; Phillips et al., 1988).

2.3.2 Synthetic chemical control methods

Various studies have reported that chemical substances such as benzoic acid and ferulic acid can reduce aflatoxins produced by *Aspergillus* species (Bilgrami et al., 1980; Chipley and Uraih, 1980; Zaika and Buchanan, 1987; Gouraman and Bullerman, 1995). Other chemical substances, which have been reported to inhibit fungal growth thus often reducing aflatoxin production by *Aspergillus* species include propionic acid, formic acids, vanillic acid, chemical treatments with ammonia, some food preservatives and caffeic acid (Chipley and Uraih, 1980; Zaika and Buchanan, 1987; Gouraman and Bullerman, 1995; Aziz et al., 1998; Jackson and Bullerman, 1999). Grains destined for animal feed are usually treated with aliphatic acid-based preservatives together with commercial products predominantly made up of propionic acid and sorbic acid salts (Magan and Aldred, 2007).

Although, there are no fungicides specifically targeting the growth of *A. flavus* and *A. parasiticus*, common fungicides used as control agents include Prochloraz, Carboxine, Bentex-T, Thiobendazole, Fernfuran, Thiram, Captan and Tebuconazole (Besri, 1992; Suberu, 2004; Mateo et al., 2017). However, fungicides such as Carboxine, Thiobendazole and Fernfuran are no longer effective in inhibiting *Aspergillus* species contamination (Besri, 1992). Other chemical fungicides used to detoxify aflatoxins from contaminated foods and feed include H_2O_2 (hydrogen peroxide), ozone, ammonia, sodium hypochlorite and sodium bisulphite (Mishra and Das, 2003). Although, these chemicals may be effective, there are harmful residues, which may be left over after detoxification, and other, more toxic compounds may be formed during detoxification (Mishra and Das, 2003). Furthermore, the chemical treatments may be

effective; however, these treatments are often not being suitable for application onto food that is intended for human consumption, which leads to the control agent being disapproved by the Food and Drug Administration (FDA) (Park et al., 1987). One example is ammonia fumigation, which has been reported to decrease the production of aflatoxins by *A. flavus* on peanuts (Park et al., 1987). However, due to the toxicity of the products produced after fumigation, the foodstuffs are unsuitable for consumption by humans (Akbas and Ozdemir, 2006).

2.3.3 Biological control methods

Biological control involves the use of competitive displacers or agents that are able to control the occurrence of a pathogen without affecting crop quality or production. A biological control strategy used against *A. flavus* and *A. parasiticus* includes non-toxigenic *Aspergillus* species, which act as competitive displacers that also reduce the incidence of the production of aflatoxins by aflatoxigenic isolates in the field (Abbas et al., 2009). In terms of aflatoxin detoxification, biological control agents, which have been used, include *Fusarium aurantiacum* (NRRLB184), *Tetrahymena pyriformis*, *Bacillus* species and *Rhizopus* species (Ciegler et al., 1966; Robertson, 1970; Cole et al., 1973; Chaurasia, 1995). These biological control agents are, however, often expensive and time consuming because the use of biological agents requires the breeding process of crop/seed varieties that are resistant to these biological control agents (Mishra and Das, 2003). Recently, the use of non-aflatoxigenic *A. flavus* is gaining popularity because previously it was applied to the soil through artificial inoculation; however, a recent study has reported its use as maize seed coatings, which are applied using starch-based bioplastic (Accenelli et al., 2018).

2.3.4 Seed treatments during storage

The preservation of grain during storage from contamination with either fungal growth or mycotoxins may consist of the use of different control method combinations, such as using a physical control method together with a chemical control method. In some studies, the evaluation of the above-mentioned control methods, mainly synthetic control methods have been evaluated. An example can be found in one study which

used neem (*Azadirachta indica*) powder and three fungicides, namely: Allette (80 % w/w), Ridomyl gold (MZ 68 % WP) and Antracol (70 % WP), on maize seeds which were artificially inoculated in conical flasks with *A. flavus*, *A. niger*, *A. wentii* and other maize fungal pathogens (Sitara and Akhter, 2007). The seeds were then treated with the above-mentioned seed treatments (Sitara and Akhter, 2007). Using the blotter method, the seed germination potential and fungal growth inhibition were evaluated (Sitara and Akhter, 2007). The growth of *A. flavus* was reported to have been reduced by the fungicide Ridomyl gold, while the fungicide, Allette was the only fungicide effective against *A. niger* growth (Sitara and Akhter, 2007).

In another seed treatment study, three allelopathic aqueous leaf extracts from the sunflower (*Helianthus annuus*), sorghum (*Sorghum bicolor*) and chinaberry (*Melia azedarach*) plants were evaluated against seed-borne *A. niger* and *A. fumigatus*, among other seed-borne fungi (Shafique et al., 2005). The three extracts were used as seed treatments on stored maize grains and then evaluated for their fungal inhibition potential. When compared to the positive control (mercuric chloride), the chinaberry extract was able to inhibit the growth of *A. fumigatus*, while the sunflower extract inhibited the growth of *A. niger* (Shafique et al., 2005).

In contrast, Boukaew et al. (2017) evaluated essential oils from *Vatica diospyroides* (vatica oil) and *Syzygium aromaticum* (clove oil) plants, which were used as maize seed treatments against *A. flavus*. The essential oils were sprayed onto artificially inoculated (*A. flavus*, 10^5 spores/mL) maize seeds (Boukaew et al., 2017). After a five-day incubation period, the fungal growth was analyzed and at the highest tested concentration (100 µL/L); both essential oils were reported to have completely inhibited *A. flavus* growth (Boukaew et al., 2017). Similarly, the protection potential of the two oils on the maize seeds was evaluated at 50 µL/L for 0, 6, 12 and 24 hours after seed treatment (Boukaew et al., 2017). After 24 hours, it was reported that both oils had exhibited complete seed protection against *A. flavus* (Boukaew et al., 2017). It was also noted that 6 and 12 hours after seed treatment; the clove oil was already protecting the maize seeds completely (Boukaew et al., 2017).

2.4 An alternative control method: Plant extracts

As a result of the many increasing side effects associated with the overuse of synthetic chemical fungicides (in an attempt to control fungal contamination of crops, grains and stored food products), there is a greater demand for natural, biodegradable, cost effective and environmentally friendlier control agents (Sahab et al., 2014; Dikhoba et al., 2019). As mentioned earlier, alternative methods which are currently being used to control fungal contaminations include physical methods such as modified atmospheres, UV treatment of grains as well as the use of biological control agents (e.g. non-aflatoxigenic strains of *Aspergillus flavus* as a competitive inhibitor) (Bluma et al., 2008a; Abbas et al., 2009). Although the abovementioned methods may be effective, these methods are not always cost effective for smallholder farmers or subsistence farmers. Therefore, plants should be evaluated for their antifungal and antimycotoxigenic properties and for their potential as biological control agents.

Medicinal plants are being evaluated for their antimicrobial activity worldwide. In South Africa, many indigenous plants have been investigated for their antimicrobial activity against bacteria and phytopathogenic fungi (Kumar and Prasad, 1992; Rabe and Van Staden, 1997; Eksteen et al., 2001; McGraw et al., 2001; Masoko et al., 2007; Lui et al., 2009; Sarcheshmeh et al., 2015; Dikhoba et al., 2019). Some of the many South African indigenous plants, which have been evaluated for their antifungal activity, include *Combretum* species, *Terminalia* species, *Merwilla plumbea*, *Artemisia afra*, *Bophane disticha*, *Bulbine frutescens*, *Crinum mocowanii*, *Dalbergia obovata*, *Dioscorea sylvatica*, *Ekebergia capensis* and *Ziziphus mucronata* (Rabe and van Staden, 1997; McGraw et al., 2001; Masoko et al., 2007; Lui et al., 2009).

2.4.1 Antimicrobial activity of plants

Plants produce secondary metabolites such as tannins, flavonoids and alkaloids, which may be biologically active against certain microorganisms such as phytopathogenic fungi (Soylu et al., 2006). The biologically active phytochemicals may thus provide potential alternatives to help curb the current problems associated with the contamination of grains, crops and stored food products (Kim et al., 2003). This is due

to the perception that plant constituents are non-toxic and sustainable when compared to synthetic chemical fungicides (Salehan et al., 2013; Dikhoba et al., 2019).

2.4.2 Plant constituents against *Aspergillus* species

The inadvertent contamination of grains, crops and stored products by *Aspergillus* species due to incorrect storage practices and poor crop management strategies result in crop yield losses (Essono et al., 2007). Moreover, the production of aflatoxins, which are potent and thermostable, make it difficult to eliminate by cooking the contaminated grains (Hasem and Alamri, 2010). The increasing awareness of natural products as food preservatives has led to the exploration of plants as alternative control agents (Schuenzel and Harrison, 2002). This may be due to some phytopathogenic fungi attaining resistance to synthetic chemical fungicides (Schuenzel and Harrison, 2002).

In the following section a brief overview of the inhibitory activity of plants and essential oils against the growth of *Aspergillus* species will be given.

Stevic et al. (2014) evaluated essential oils extracted from fifteen plants, namely: savory (*Satureja hortensis*), orange (*Citrus amara*), thyme (*Thymus vulgaris*), rose (*Rosa damascena*), geranium (*Pelargonium graveolens*), lavender (*Lavandula angustifolia*), chamomile (blue) (*Matricaria recutita*), bergamot (*Citrus bergamia*), lemon (*Citrus limon*), oregano (*Origanum heracleoticum*), eucalyptus (*Eucalyptus globulus*), anise (*Illicium verum*), tea tree (*Melaleuca alternifolia*) and viola (*Viola odorata*) against the growth of *A. flavus* and *A. niger*. Furthermore, MIC values reported to be less than 1 mg/mL against *A. flavus* growth were those from savory, orange, rose and geranium essential oils (Stevic et al., 2014). In the same study, all the essential oils were evaluated for their antifungal activity against *A. niger*. The MIC values obtained, which were less than 1 mg/mL were those from savory, orange, thyme, rose, oregano and eucalyptus essential oils (Stevic et al., 2014). Furthermore, it was noted that the essential oils exhibited better antifungal activity against *A. niger* when compared to *A. flavus* (Stevic et al., 2014). Overall, the best (< 0.30 mg/mL) essential oils against both *Aspergillus* species were essential oils extracted from savory and oregano (Stevic et al., 2014).

However, in another study aqueous extracts from *Thymus vulgaris* were evaluated for their growth reduction potential against *A. flavus* (Sarcheshmeh et al., 2015). The MIC value (200 µg/mL) obtained was lower than the MIC value (0.28 mg/mL) obtained in the study conducted by Stevic et al. (2014), which used thyme essential oils instead of extracts (Sarcheshmeh et al., 2015).

In a study focusing on the antifungal activity of a crude extract obtained from *Nitraria schoberia* fruit against *A. niger*, the fruit extract concentrations of 50, 100, 150, 200, 250 and 300 µg/mL, were investigated using the disc diffusion method where the diameters of the zones of inhibition were recorded (Sharifi-Rad et al., 2015). The diameter of the zones of inhibition increased with increasing extract concentration (Sharifi-Rad et al., 2015). The zones of inhibition at the 250 and 300 µg/mL showed better inhibition (15.70 and 18.20 mm, respectively) when compared with the positive control, ketoconazole (14.7 mm) (Sharifi-Rad et al., 2015).

Methanol extracts of *Satureja hortensis* were used to determine the wet and dry mycelial weight of *A. flavus* grown in extract amended liquid media (Dikbas et al., 2008). At extract concentrations of 25, 12.50 and 6.25 µL/mL, the wet and dry mycelial weights were reported to be 11.06, 12.53 and 14.56 g, respectively, while the dry weights reported were 3.26, 3.90, 5.10 g, respectively (Dikbas et al., 2008). The wet and dry mycelial weights of *A. flavus* in extract amended liquid media were compared to the untreated wet (12.60 g) and dry (3.86 g) mycelial weight of *A. flavus*, where it was noted that as the extract concentration increases, there was a decrease in the mycelia weight of the fungus (Dikbas et al., 2008).

2.4.3 Reduction of aflatoxin production by plant constituents

Certain *Aspergillus* species, including *A. flavus*, *A. parasiticus*, *A. nomius*, *A. ochraceus* and *A. bombycis* produce aflatoxins (Cotty et al., 1994; Klich et al., 1998; Peterson et al., 2001; Ehrlich et al., 2007). Similarly, only certain aflatoxigenic strains are able to produce significant amounts of aflatoxins (Chipley and Uraih, 1980). This section will focus on the anti-aflatoxigenic potential of plant constituents.

The antifungal and anti-aflatoxigenic activities of methanolic *Allium sativum*, *Lantana camara*, *Olea europaea*, *Punica granatum* and *Zingiber officinale* extracts were evaluated against *A. flavus* isolated from *Triticum* spp. seeds (Mostafa et al., 2011). The effect of the plant extracts on the fungus at a concentration of 10 mg/mL was investigated using the poisoned food method (Mostafa et al., 2011). Among the tested extracts, *P. granatum* was reported to have the best growth inhibition properties (91.58 %) when compared to the other tested extracts (Mostafa et al., 2011). Furthermore, the production of aflatoxins was evaluated by correlating the production of aflatoxins with fungal growth (Mostafa et al., 2011). The extracts were reported to have completely inhibited the production of AFB_1 at extract concentrations of 5 mg/mL for *P. granatum*, 10 mg/mL for *Z. officinale* and 15 mg/ml for *O. europaea* (Mostafa et al., 2011). Moreover, an extract concentration of more than 25 mg/mL was required for *L. camara* to inhibit *A. flavus* growth and AFB_1 production (Mostafa et al., 2011). Conversely, *A. sativum* was reported to have no inhibitory activity against both *A. flavus* growth and aflatoxin production (Mostafa et al., 2011).

In another study, essential oils extracted from fennel (*Foeniculum vulgare*), coriander (*Corandrum sativum*), caraway (*Carum carvi*), rosemary (*Rosmarinus officinalis*), basil (*Ocimum basilicum*) and peppermint (*Mentha×piperita*) were evaluated for their growth and aflatoxin inhibition properties against *A. flavus* (Deabes et al., 2011). The essential oils were evaluated at concentrations of 500, 750 and 1000 parts per million (ppm) (Deabes et al., 2011). The basil, rosemary, coriander and caraway essential oils exhibited complete fungal growth inhibition at an essential oil concentration of 1000 ppm (Deabes et al., 2011). The production of aflatoxins, quantified using HPLC, determined that at all essential oil concentrations of basil and coriander inhibited aflatoxin production by *A. flavus* (Deabes et al., 2011). The percentages of aflatoxin inhibition by basil and coriander reported were 24.91, 23.80 and 23.88 %, respectively, while the aflatoxin inhibition percentages for coriander reported were 22.96, 25.13 and 25.38 %, respectively (Deabes et al., 2011).

Similarly, aqueous extracts from *Allium cepa*, *A. sativum*, *Aloe vera*, *Cassia italica*, *C. sativum*, *Eucalyptus globulus*, *Olea europaea*, *Thymus vulgaris*, *Z. officinale* and

Zizyphus spina were evaluated for their anti-aflatoxigenic activity (El-Aziz et al., 2012). The extracts were added into SMKY (Sucrose, Magnesium Sulphate, Potassium Nitrate, Yeast extract) liquid medium to obtain medium concentrations of 5, 10, 15 and 20 % (El-Aziz et al., 2012). The percentage of aflatoxin inhibition for each plant extract was calculated after the toxins were quantified using HPLC for 25 minutes (El-Aziz et al., 2012). At an extract concentration of 20 %, *T. vulgaris* and *Z. officinale* were able to inhibit 79.10 % AFB_1 production, while *O. europaea* and *E. globulus* were able to inhibit AFB_1 production by 75 % at the same extract concentration (Al-Aziz et al., 2012). Similarly, *T. vulgaris* and *O. basilicum* were able to inhibit 76.20 % AFB_2 production by *A. flavus* (El-Aziz et al., 2012). However, *Z. spina* and *C. italica* exhibited minor effects inhibiting AFB_1 and AFB_2 production (16.60 and 9.50; and 25 and 23.80 %, respectively) (Al-Aziz et al., 2012).

Murray koenigii, *Ocimum basilicum*, *Phyllanthus emblica*, *Terminalia bellerica*, *T. chebula* and *Z. officinale* extracted with methanol-water (2:1 v/v) were evaluated for their aflatoxin production reduction properties in artificially inoculated broth (Shukla et al., 2012). The extracts were added into semisynthetic SMKY broth medium to obtain a 1 mg/mL growth medium concentration (Shukla et al., 2012). After a 10-day incubation period, the broth was filtered and extracted of AFB_1 using chloroform (Shukla et al., 2012). Thin layer chromatography and spectrophotometry were used to detect and quantify the presence of AFB_1 in the filtrates (Shukla et al., 2012). The aflatoxin content present in the growth medium was calculated using the obtained optical density of the samples and reported to be 11.30, 10.30, 0.0, 12.30, 0.00 and 7.60 µg/g of AFB_1, when compared to the control, which had 19.80 µg/g (Shukla et al., 2012). Furthermore, it was reported that the plant extracts exhibited better activity as anti-aflatoxigenic agents when compared to fungal growth inhibition activity (Shukla et al., 2012).

2.4.4 Seed treatments with plant extracts

Plant extracts consist of a combination of natural plant metabolites (primary) and metabolites produced for defense against plant pests (secondary) (Compean and Ynalvez, 2014). Primary metabolites are essential for plant metabolism and growth, while secondary metabolites that are used in plant defense mechanisms are produced

as a result of primary metabolism (Ramawat, 2007; Compean and Ynalves, 2014). Due to the presence of these combined compounds, plant extracts have the potential to be used as seed protectants against seed pests and/or as seed growth or germination enhancers as reported in the following studies below.

Basra et al. (2011) evaluated the treatment of hybrid maize seeds with different moringa (*Moringa oleifera*) leaf extracts (1:30, 1:40 ethanol: water) as seed priming agents. The maize seeds were primed with the leaf extracts, while the control seeds were treated with water, for 18 hours then dried on filter sheets for 48 hours at room temperature (Basra et al., 2011). The seeds were then sown and evaluated for the emergence and seedling vigor (Basra et al., 2011). Basra et al. (2011) reported that the moringa extracts enhanced the rate of seed germination by 6 % compared to the seeds treated with water only.

In contrast, Usha-Rani and Devanad (2011) investigated the protection potential of four plant extracts on maize seeds against *Sitophilus oryzae* and *Tribolium castaneum*. The ethyl acetate-extracted extracts, at extract concentrations ranging between 15 and 150 µg/seed, were applied onto the maize seeds while the control seeds were treated with ethyl acetate only (Usha-Rani and Devanad, 2011). The viability of the seeds was evaluated after 90 days of storage and exposure to the two seed pests (Usha-Rani and Devanad, 2011). According to Usha-Rani and Devanad (2011), the seed treatments protected the seeds as 100 % of *S. oryzae* and more than 90 % of *T. castaneum* pests were killed after 72 hours of exposure to the treated seeds.

Similarly, Kelli et al. (2018) used methanolic extracts of *Cestus incants* on artificially inoculated macadamia nuts to inhibit aflatoxin B_1 production by *A. parasiticus*. The treated macadamia nuts were incubated in flasks containing YES (yeast extract sucrose) media and 100 µL of the extract for 15 days at 30 °C (Kelli et al., 2018). Kalli et al. (2018) reported that at 0.2 g/mL, the growth of *A. parasiticus* was inhibited by 46 % while the production of AFB_1, quantified using HPLC, was reduced by 90 % after 15 days, when compared to the control (AFB_1: 0.53 µg/g for the extract and 5.14 µg/g for the control).

In a study by Sharma and Sharma (2012) the evaluation of the antifungal activity and the seed protection potential of three leaf extracts against *A. flavus* and *A. parasiticus* growth and aflatoxin production were done. The aqueous extracts of *Lawsonia inermis* and *Murraya paniculata* had minimum inhibitory concentration (MIC) values of 5 and 8 mg/mL, respectively, against *A. flavus* (Sharma and Sharma, 2012). However, against *A. parasiticus*, *L. inermis* and *M. paniculata* had MIC values 10 and 9 mg/mL, respectively (Sharma and Sharma, 2012). The extracts of the two plants (at 10 and 100 mg/g concentrations) were then used as seed protectants on freshly harvested maize seeds (Sharma and Sharma, 2012). The treated seeds were artificially inoculated with a standard 285 × 10^4 spores/mL suspension of *A. flavus* and *A. parasiticus*, then dried and packaged in plastic containers, which were stored for six months (Sharma and Sharma, 2012). Furthermore, it was reported that the inhibitory activity of the extracts was 2 to 3-fold higher for the stored products compared to the MIC values obtained, which were 33-40 mg/mg and 40-45 mg/g against *A. flavus* and *A. parasiticus*, respectively (Sharma and Sharma, 2012). The production of aflatoxins, quantified using HPLC, was reported to have been reduced by 75 % for both *A. flavus* and *A. parasiticus* (Sharma and Sharma, 2012).

Comparably, ten aqueous plant extracts were evaluated as stored maize seed protectants against *A. flavus* growth (Iram et al., 2018). Artificially inoculated and plant extract (10 % extract concentration v/v) treated seeds were incubated for four days at 28 °C (Iram et al., 2018). The percentage growth inhibitions of the three best extracts on coconut-based media were reported to be 100 % (*Eucalyptus citriodora*), 91 % (*Trachyspermum ammi*) and 83 % (*Ocimum basilicum*) (Iram et al., 2018).

2.5 Overview of the selected plants for this study

The selected plants for this study were chosen based on literature available on their antimicrobial activities. The selected plants are *Erythrophleum lasianthum*, *Heteropyxis natalensis* and *Warburgia salutaris*. In the following section a brief overview of the plant, as well as its medicinal properties will be given. The antimicrobial activity of the extracts made from these plants will be emphasized.

2.5.1 Background on selected plants

Erythrophleum lasianthum Corbishely (Fabaceae)

Erythrophleum lasianthum (Figure 2.5) is also known as the Maputaland ordeal tree (Watt and Beyer-Brandwijk, 1962; Palmer and Pitman, 1972). It is a medium to large tree that can grow up to 17 meters tall (SANBI, 2017). The bark has a rough greyish-brown surface (SANBI, 2017). The leaves have alternating smaller leaflets that can grow up to 40 × 20 mm (SANBI, 2017). The flowers are often a greenish-yellow to cream colour, that later develops into flat, brown and woody pods (fruit) that have lens-shaped seeds (SANBI, 2017). The Maputaland ordeal trees grows mainly in the Grassland and Savanna biomes, and are predominantly distributed in the KwaZulu-Natal province, eSwatini (formerly known as Swaziland) and Mozambique (Williams et al., 2008). It is near threatened due to severe bark harvesting for medicinal plant trading and because of its occurrence outside protected areas of growth due to habitat loss (Williams et al., 2008).

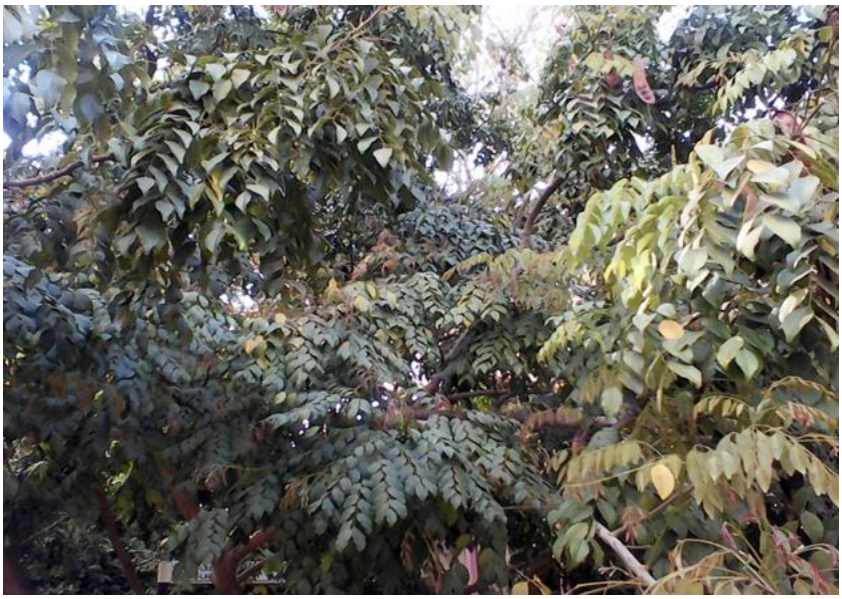


Figure 2.5: *Erythrophleum lasianthum* (Photo credit: S.H. Chauke)

This plant is traditionally used as treatment for abdominal pains and as an anthelmintic (Watt and Beyer-Brandwijk, 1962; Palmer and Pitman, 1972). However, certain parts of

the tree, such as the bark, are taken in a powder form as snuff for headaches (Hutchings et al., 1996).

***Heteropyxis natalensis* Harv. (Heteropyxidaceae)**

Heteropyxis natalensis (Figure 2.6), commonly known as the Lavender tree is not endemic to South Africa; however, it occurs mainly in the Gauteng, Limpopo, Mpumalanga and KwaZulu-Natal provinces (Foden and Potter, 2005). It also occurs in Zimbabwe and eSwatini (SANBI, 2002). It is currently not threatened nor endangered (Foden and Potter, 2005). The lavender tree is a deciduous tree that grows 4 to 10 meters tall (SANBI, 2002). The stems or branches of the lavender tree droop with narrow, elliptic and spirally arranged leaves (Palgrave, 1977; Pooley, 1993; Van Wyk and Van Wyk, 1997). The leaves are a dark green colour on top and a slightly paler green at the bottom (SANBI, 2002). The crushed leaves emit a strong lavender scent (Foden and Potter, 2005). The bark is flaky and pale grey to white in colour (Palgrave, 1977; Pooley, 1993; Van Wyk and Van Wyk, 1997). In summer, the flowers are a yellow-green colour, small and less than 3 mm in diameter (SANBI, 2002). The fruits are also small and capsule shaped (SANBI, 2002).

Figure 2.6: *Heteropyxis natalensis* (Photo credit: S.H. Chauke)

Heteropyxis natalensis is often incorporated in perfumes due to its camphor-like odour when the twigs or leaves are crushed (Gundidza et al., 1993). It is traditionally used for making medicinal teas for the treatment of bleeding gums (Gundidza et al., 1993).

***Warburgia salutaris* (G. Bertol). Chiov. (Canellaceae)**

Warburgia salutaris (Figure 2.7), otherwise known as the Pepper-bark tree, is widely distributed, in the KwaZulu-Natal, Limpopo and Mpumalanga provinces of South Africa (Williams et al., 2008; Maroyi, 2014). It does, however, also occur in eSwatini, Mozambique, Malawi and Zimbabwe (Williams et al., 2008). It is often found growing in thickets, coastal areas and sandy to desert-like forests (SANBI, 2004). Due to excessive harvesting of *W. salutaris* bark for traditional medicinal uses, its population in South Africa has declined by at least 50%, especially in KwaZulu-Natal (Williams et al., 2008). Although, *W. salutaris* extinctions and very low population numbers have been documented, some sub-populations still occur in the Limpopo and Mpumalanga provinces, with majority of this plant species being targeted mainly by bark harvesters (Williams et al., 2008).

Figure 2.7: *Warburgia salutaris* leaves (Photo credit: S.H. Chauke)

The Pepper-bark tree is an evergreen tree that can grow 5 to 10 metres tall (Palgrave, 1977; Pooley, 1993; Van Wyk and Van Wyk, 1997). The bark is covered in reddish-brown spots (Palgrave, 1977; Pooley, 1993; Van Wyk and Van Wyk, 1997). The leaves are arranged in an alternating manner with a glossy dark green colour on top

and a lighter shade of green at the bottom (Palgrave, 1977; Pooley, 1993; Van Wyk and Van Wyk, 1997). The flowers are small and white to greenish in colour and can grow up to 7 mm in diameter (SANBI, 2004). During winter and early summer (July to December), the flowers develop into oval-rounded berries that become purple as they ripen (SANBI, 2004).

Its traditional uses include the treatment of blood disorders, cancer, chest complaints, constipation, diabetes, diarrhoea, inflammation, backaches, pneumonia and skin sores (Watt and Beyer-Brandwijk, 1962; Gelfand et al., 1985; Mabogo, 1990; Hollmann and van der Schijff, 1996; Hutchings et al., 1996; Felhaber and Mayeng, 1997; Mukamuri and Kozanayi, 1999; Rabe and Van Staden, 2000; Deutschlander et al., 2009).

2.5.2 Bioactivity and compounds isolated from the selected plants

Plant extracts are made up of complex chemicals, which often have antimicrobial effects on certain microorganisms (Schmourlo et al., 2005). The separation and isolation of antimicrobial compounds is generally a limiting obstacle due to the complex chemical compounds present in plant extracts (Schmourlo et al., 2005).

This section will focus on the antimicrobial activity of the extracts, essential oils and isolated compounds from the selected plants.

2.5.2.1 *Erythrophleum lasianthum*

The antimicrobial activity of *E. lasianthum* is not well studied, however, its activity has been investigated against *Candida albicans* and *Mycobacterium smegmatis* (Nielsen et al., 2012). The antimicrobial activity of the methanolic leaf extracts (using the broth microdilution method) against *C. albicans* and *M. smegmatis* were reported to have MIC values of 625 µg/mL for both microorganisms, while the methanolic bark extracts were reported to be 312.50 and 625 µg/mL, respectively (Nielsen et al., 2012). The toxicity of *E. lasianthum* against these microorganisms was found to be due to two diterpene alkaloids and a phenolic glucoside (Orsini et al., 1997). The isolated compounds were identified as 3-hydroxynoerythrosuamine and 3-O-β-D-glucopyranoside and its phenolic glucoside, reseveratrol-3-β-D-glucopyranoside (Orsini et al., 1997).

2.5.2.2 *Heteropyxis natalensis*

The antimicrobial activity of the lavender tree has been investigated for its activity against oral bacteria such as *Actinomyces israeli*, *Prevotella intermedia*, *Streptococcus mutans*, *Lactobacillus paracasei* and the fungus *C. albicans* (Henley-Smith et al., 2018). Its activity against these oral bacteria has been attributed to the presence of compounds which have been isolated from it, such as cardamomin, 5-hydroxy-7-methoxy-6-methylflavanone, aurentiacin, quercetin and 3, 5, 7-trihydroxyflavan (Henley-Smith, 2018). In a study by Adesanwo et al. (2009) a chalcone compound identified as (E)-1-(2', 4'-dihydroxy, 5'-methoxy, 3'methylphenyl)-3-phenylprop-2-en-1-one was isolated from *H. natalensis*. However, the activity of the plant and compounds isolated from it, have not been investigated against other microorganisms.

The ethanolic leaf extracts were investigated against oral microorganisms (as mentioned above) using the broth microdilution method (Henley-Smith et al., 2018). The only noteworthy (≤ 1 mg/mL) MIC value obtained was against *A. israelii* (0.88 mg/mL) (Henley-Smith et al., 2018).

The antifungal activity of essential oils made from *H. natalensis* leaves were evaluated against *A. flavus, A. niger*, *A. ochraceus* and *A. parasiticus*, using the poisoned food method (Gundidza et al., 1993). The fungal growth reduction percentages reported were 99.20, 99.00, 99.10 and 97.30 %, respectively (Gundidza et al., 1993).

2.5.2.3 *Warburgia salutaris*

The pepper-bark tree has been screened against many microorganisms to investigate and evaluate its biological activity and its inhibitory effect. The bark and leaf extracts of *W. salutaris* have been reported to inhibit the growth of *Escherichia coli, Staphylococcus aureus*, *Bacillus subtilis* and *Micrococcus luteus* (Rabe and van Staden, 1997; Zschocke et al., 2000; Mohanlall and Odhav, 2009). Its antibacterial activity against *S. aureus*, *B. subtilis* and *M. luteus* was reported as 12.50, 12.50 and 50 µg/mL, respectively (Rabe and Van Staden, 2000).

Acetone leaf and bark extracts of *W. salutaris* were investigated against five *Fusarium* species, namely: *F. verticillioides*, *F. oxysporum*, *F. nygamai*, *F. graminearum* and *F. proliferatum* (Samie and Mashau, 2013). Minimum inhibitory concentration (MIC) values obtained using the broth microdilution method for the bark extracts were all ≥ 7.50 mg/mL except against *F. nygamai* and *F. proliferatum* (3.75 mg/mL), while the MIC values obtained for the leaf extracts were all > 7.50 mg/mL except against *F. nygamai* (0.48 mg/mL) and *F. graminearum* (0.95 mg/mL) (Samie and Mashau, 2013).

In another study, dichloromethane-methanol and water leaf and bark extracts, were investigated against *S. aureus*, Methicillin-resistant *S. aureus* (MRSA), gentamycin-resistant *S. aureus* (GMRSA), *S. epidermidis*, *Brevibacillus agri*, anaerobic *Propionibacterium acnes*, *Pseudomonas aeriginosa*, *Trichophyton mentagrophytes*, *Microsporum canis* and *C. albicans* (Mabona et al., 2013). Among all the extracts, the bark extracts exhibited greater antimicrobial activity when compared to the leaf extracts (Mabona et al., 2013). Noteworthy (< 1.0 mg/mL) MIC values were reported for the dichloromethane-methanol bark extract against *S. aureus*, MRSA and *T. mentagrophytes* (Mabona et al., 2013). Similarly, the dichloromethane-methanol leaf extract exhibited noteworthy activity against *S. aureus*, GMRSA, *S. epidermidis*, *P. aeruginosa* and *T. mentagrophytes* (Mabona et al., 2013). Overall, the leaf and bark extracts were reported to have moderate (< 8.0 mg/mL) antimicrobial activity against the test microorganisms (Mabona et al., 2013).

In various other studies, the antimicrobial activity of *W. salutaris* was attributed to the presences of compounds such as drimane and colorotane sesquiterpenes, tannins and mannitol (Watt and Breyer-Brandwijk, 1962; Jansen and De Groot, 1991; van Wyk and Gericke, 2000; Frum et al., 2005; Frum and Viljoen, 2006). Some of the drimane sesquiterpenes that have been isolated, identified and reported to contribute to the antimicrobial activity of *W. salutaris* include 11α-hydroxycinnamosmolide, isopolygodial (also known as isotadeonal), warburganal, polygodial, salutarisolide, muzigadial (also known as cannelal), cinnamodial (ugandensidial), isopolygodial, mukaadial, isodrimenol and monoaldehyde polygodial (Mashimbye, 1993; Mashimbye et al.,1999a; Mashimbye

et al., 1999b; Rabe and van Staden, 2000; Madikane et al., 2007). In a study conducted by Monhanlall and Odhav (2009), a sesquiterpenoid identified as 5, 10-dihydro-6, 7-dimethyl-4H-benzo [5, 6] cyclophepta [1,2b]-furan was isolated from the bark of *W. salutaris*.

Some of the isolated compounds such as drimane sesquiterpenoid lactone, identified as 11α-hydroxycinnamosmolide, were reported to have anti-mycobacterial activity against *Mycobacterium bovis* and *Mycobacterium tuberculosis* (Madikane et al., 2007). In other pharmacological reports, *W. salutaris* has been reported to show inhibitory activity against 5-lipoxygenase and cyclooxygenase-1-enzyme (anti-inflammatory properties) (Zschocke et al., 2000; Frum and Viljoen, 2006). Its contribution to anti-inflammatory activity includes inducing inflammatory cytokine expression, which is responsible for cell signaling, which in turn leads to the release of infection fighting cells towards the affected area (Frum and Viljoen, 2006; Leshwedi et al., 2008). Therefore, the extracts are traditionally used topically onto the skin for the treatment of skin diseases as previously mentioned (Frum and Viljoen, 2006). Compounds isolated from *W. salutaris* such as mukadiaal and warburganal were reported to have anti-inflammatory and antioxidant activities by offering protection against inflammatory induced effects such as lipid peroxidation and DNA strand breakage (Leshwedi et al., 2008).

2.6 Background on the crop plant: *Zea mays*

Maize (*Zea mays*) is an annual grass in the Poaceae family (Department of Agriculture, Forestry and Fisheries, 2018). It is a staple crop for 200 million people, which is almost a quarter of the world's population (Brown et al., 1988). This is because maize is the third major cereal grain produced in the world, after rice (*Oryza sativa*) and wheat (*Triticum aestivum*) (Ekwomadu et al., 2018). Due to the diverse functionality of maize, its popularity has increased as a food source worldwide for both humans and animals (Nuss and Tanumihardjo, 2010). This is because maize can be consumed and prepared in various ways including being boiled, roasted, ground to a powder and used for porridge as well as being processed to form thickeners, sweeteners and non-consumables (Inglett, 1970; Whistler, 1970). Therefore, maize is an important international trade commodity and the rate at which it is consumed is increasing

annually (Du Plessis, 2003; Abassian, 2006; Bassapa, 2009; O'Gara, 2007). According to estimates made by the FAO (Food and Agriculture Organization of the United Nations), the demand for maize consumption by humans and animals will increase to 300 million tons by 2022 (FAOSTAT, 2012). Approximately, of the total maize produced worldwide, 65 % is used as animal feed, 15 % is used for human consumption/food and 20 % is intended for industrial use(s) (Abassian, 2006).

In most sub-Saharan African households, maize is one of the main sources of food and crop income (Ng'ang'a et al., 2016). Approximately, 40 % of the total dietary intake in the eastern and southern African countries comprises of maize as a food source (Doss et al., 2003; Kimanya et al., 2008; Ng'ang'a et al., 2016). Furthermore, it was estimated that a person's maize consumption in South Africa can reach up to 400 grams daily, in the form of either processed foods such as breakfast cereals, maize meal or snacks or directly consumed as corn (Sydenham et al., 1991; Shephard et al., 2007).

2.6.1 Maize production in South Africa

Maize is a summer crop that is widely cultivated, and the largest grain crop produced in South Africa (Du Plessis, 2003; DAFF, 2018). On average, approximately 8 million tons of maize is produced annually (DAFF, 2017). In 2018, approximately 12 827 million tons of commercial maize was planted, which is 23.70 % (or 3 993 million tons) less than the commercial maize produced in 2017 (16 820 million tons) (DAFF, 2018). As shown in Figure 2.8, the Free State province, which is the major producer of maize in South Africa produces 44 %, while the North West and Mpumalanga provinces produce 19 and 20 %, respectively (DAFF, 2018). An estimated area of 2 319 million hectares is expected to yield 5.53 tons (hectare of maize in the first quarter season of 2018) (DAFF, 2018). The North West province, which is one of the top three maize producing provinces in South Africa, has the highest recorded maize fungal contamination cases when compared to other South African provinces (Ekwomadu et al., 2018). Therefore, since majority of the maize producers in the North West province are small-scale farmers, fungal contamination of the crop is of great concern (Ncube and Flett, 2012).

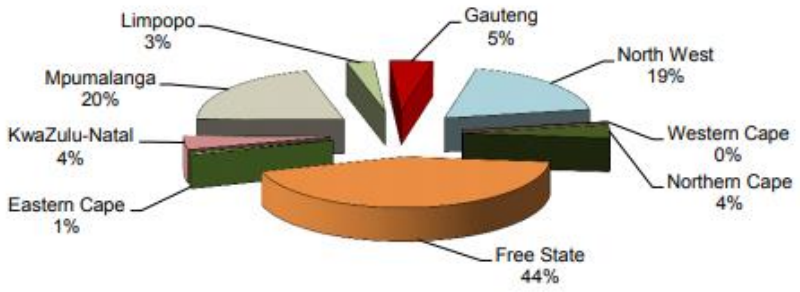


Figure 2.8: Maize production distribution in South Africa (DAFF, 2018).

Similarly, in other African countries, small-scale farmers, produce the bulk of maize grains, however, due to annual resource constraints in countries such as Kenya, less than 5 ha is cultivated, yearly (Ng'ang'a et al., 2016).

2.6.2 Contamination of maize with fungi and mycotoxins

The loss in yield of maize harvested, the decrease in the grain quality and its economic value, is due to various factors, which occur before, during and after harvesting (Cotty et al., 1994; The World Bank, 2010). One of the primary causes for this loss is due to fungal infection of maize, which can also occur at any stage of development, cultivation, harvesting and during storage (Ng'ang'a et al., 2016). Some of these pathogenic fungi include *Aspergillus* spp., *Penicillium* spp., *Alternaria alternata* and *Fusarium* spp. (Quezada et al., 2006; Blandino et al., 2009; Chulze, 2010). Although some of these fungi infect various parts of the plant, the greatest concern to public health is the production of mycotoxins by certain fungal species from the *Aspergillus*, *Fusarium* and *Penicillium* genera (Gong et al., 2004). These fungi produce various mycotoxins such as aflatoxins, ochratoxin A, sterigmatocystin, patulin A, gliotoxin, citrinin (*Aspergillus* spp.), cyclopianozonic acid (*Penicillium* spp.), fumonisins, trichothecenes, deoxynivalenol, moniliformin and zealarenone (*Fusarium* spp.), which are a threat to human, animal and plant health (Cole et al., 1973; Abbas et al., 1995; Gong et al., 2004; Ismaiel and Papenbrock, 2015).

Concerning aflatoxins, an outbreak of aflatoxicosis was reported in Kenya in 2005 due to the ingestion of aflatoxin-contaminated maize (Azziz-Baumgarter et al., 2005; Lewis et al., 2005). Another mycotoxin-related foodborne incident occurred in India, where people who consumed mouldy sorghum and maize contaminated with fumonisin B_1 were reported to have suffered abdominal pain and had diarrhea (Bhat et al., 1997).

2.6.3 Occurrence of *Aspergillus flavus* and aflatoxin B_1 on maize grains produced in South Africa

The contamination of maize grains with *A. parasiticus* has not been reported on South African maize grains, however, contamination with *A. parasiticus* has been reported in countries such as Zambia, Romania, France, Hungary, Argentina and North-east Italy (Piva et al., 2006; Garrido et al., 2012; Battilani et al., 2013; 2016; Kachapulula et al., 2017). The following section will focus on the contamination of maize grains with Aspergillus flavus and aflatoxins specifically in South Africa.

The contamination of maize grains with *A. flavus* reduces the crop yield, which is originally reduced by heat and drought stress (PAS, 2010). *Aspergillus flavus* infected and aflatoxin contaminated grains are often discarded or deemed unsuitable for consumption if the aflatoxin content exceeds the prohibited amount (in South Africa) of 10 µg/kg of aflatoxins, 5 µg/kg of AFB_1 for all foodstuffs and 15 µg/kg for peanuts and milk (PAS, 2010; CANSA, 2017). This is because most effective detoxification methods are often associated with the negative perceptions of the public due to either the high production costs of the synthetic fungicides and/or chemical preservatives, their long-term effects on the environment or the fungi building resistances to current control agents (Eckert and Ogawa, 1988; Dikbas et al., 2008; PAS, 2010).

Mngqawa et al. (2016) collected and analyzed stored maize from 20 different subsistence farms from villages in the Gert Sibande District Municipality in Mpumalanga (GSDM) and 19 farms from the villages in the Vhembe District Municipality in the Limpopo province (VDM) for mycotoxin contamination using LC-MS/MS (Mngqawa et al., 2016). These stored grain samples were randomly collected from farmers six weeks after the crops had been harvested in July 2011 and July 2012

(Mngqawa et al., 2016). The 29 samples were analyzed for the occurrence of aflatoxins among other mycotoxins such as fumonisins (Mngqawa et al., 2016). Twenty-one percent of the maize grains collected from VDM tested positive for the presence of aflatoxins (1-149 µg/kg) in 2011 and 30 % in 2012 (1-144 µg/kg) (Mngqawa et al., 2016). In the samples collected from GSDM, none of the maize samples were contaminated with aflatoxins in 2011 (Mngqawa et al., 2016). However, in 2012, 32 % of the samples were contaminated with aflatoxins (1-39 µg/kg) (Mngqawa et al., 2016). In most of the contaminated samples, AFB_1 attributed to the highest aflatoxin percentage ranging between 1-133 µg/kg (2011) and 1-73 µg/kg (2012) (Mngqawa et al., 2016). Approximately, 62 % of the AFB_1 contaminated grains collected from VDM contained levels above the South African regulations limit of aflatoxins in all food stuff (5 µg/kg of AFB_1 and aflatoxin total of 10 µg/kg) (Rheeder et al., 2009; Mngqawa et al., 2016).

Similarly, in food commodities including maize grains, obtained from a grain market in Durban, South Africa, Olagunju et al. (2018) reported that 85.50 % of the total analyzed samples of the grains were contaminated with *Aspergillus* species. From the analyzed samples, the Bambara groundnuts were all (100 %) contaminated with *Aspergillus* spp. while the spices, rice and maize grains along with their product derivatives were only contaminated with 89.50, 86.90 and 71.90 % of *Aspergillus* species, respectively (Olagunju et al., 2018). It was further noted that *A. flavus* was more prevalent in the groundnut (64.70 %) and spice (44.70 %) samples (Olagunju et al., 2018).

A similar study evaluating maize grain products for fungal and mycotoxin contamination, was done by Adekoya et al. (2018), on five of some of the most popular fermented food products sold in food markets in Johannesburg and Pretoria. It was noted that the *Aspergillus* genus was the dominating fungal genus contaminating all the analyzed foodstuffs namely: locust beans (meat substitute), maize meal alcoholic beverage, fermented melon, African oil bean (consumed in the form of porridge) and maize gruel (weaning food) (Adekoya et al., 2018). The maize meal alcoholic beverage sample was reported to have 37 % more AFB_1 content when compared to the other analyzed samples (Adekoya et al., 2018).

2.7 Conclusions and Limitations

The control of aflatoxigenic *Aspergillus* species, in terms of their growth and ability to produce aflatoxins on stored grains or in the field, requires formulations or plant-derived compounds, which can specifically inhibit or suppress fungal growth and/or the production of aflatoxins. This literature review provided a brief summary of the conditions favourable for the growth of *A. flavus* and *A. parasiticus* and the production of aflatoxin B_1. Furthermore, literature on the antifungal and anti-aflatoxigenic potential of plant-derived products such as plant extracts and essential oils was evaluated. There are other methods of control, which are often effective, however, most of these methods are not always suitable for food application or are often expensive to maintain and moreover, not environmentally friendly.

Therefore, what is lacking from the literature is the evaluation of South African indigenous plants as antifungal and anti-aflatoxigenic control agents *in vitro*. Furthermore, research into identifying and isolating compounds present in plants with the ability to inhibit the fungal growth and the production of aflatoxins by *Aspergillus* species is needed. Similarly, their efficacy as potential seed treatments against fungal and mycotoxin contamination on maize grains needs to be evaluated. Literature is also lacking on the evaluation of the potential of South African indigenous plants as target-specific fungicides or fungicide enhancers against *Aspergillus* species, and studies on their cytotoxicity is also not available.

Chapter 3
In vitro assessment of selected South African indigenous plants on aflatoxigenic *Aspergillus* species and their cytotoxicity

3.1 Introduction

Aspergillus flavus and *A. parasiticus* are teleomorphic saprophytes, which grow naturally on various substrates such as plant and animal debris, drought stressed crops and in the soil in the field and in climatic (humid and warm) conditions suitable for fungal growth (Gourama and Bullerman, 1995, Bhatnagar et al., 2000; Scheidegger and Payne, 2003). In addition, *A. flavus* and *A. parasiticus* grow on insect damaged, poorly stored grain and on grains stored at temperatures ranging between 25 and 35 °C (Lisker and Lillehoj, 1991; Klich, 2002; Zablotowicz et al., 2007). Apart from being pathogenic to plants and stored grains, *A. flavus* and *A. parasiticus* produce very potent mycotoxins, known as aflatoxins (Klich, 2007a). These polyketide-derived secondary metabolites are not only mutagenic and hepatotoxic, but they are also teratogenic (Eaton and Groopman, 1994; Horn, 2007; Turner et al., 2013). There are four main types of aflatoxins namely: aflatoxin B_1 (AFB_1), aflatoxin B_2 (AFB_2), aflatoxin G_1 (AFG_1) and aflatoxin G_2 (AFG_2), along with their derivatives formed in milk (aflatoxin M_1 and M_2) (Horn et al., 1996). Amongst the above-mentioned aflatoxins, AFB_1, which is produced mainly by *A. flavus* and *A. parasiticus*, is the most toxic, is carcinogenic, associated with childhood stunting and can act as an immunosuppressor (Eaton and Groopman, 1994; Klich, 2007a; Reddy et al., 2010). Aflatoxin B_1 can cause aflatoxicoses and in severe cases, liver toxicity in humans and animals, upon accumulated ingestion of contaminated foods and feed (Hedayati et al., 2007; Amaike and Keller, 2011). Moreover, in plants, aflatoxins have been reported to limit plant growth through

inhibiting seedling growth, seed germination and through interrupting physiological processes such as those, which produce photosynthetic pigments (Prasad et al., 1996).

Current control methods against *A. flavus* and *A. parasiticus* contamination of foods and feed include physical methods, such as the use of modified atmospheres, synthetic chemical control methods (fungicides and food preservatives) as well as the use of a non-toxigenic competitive displacer such as non-toxigenic *Aspergillus* species (biological control) (Bluma et al., 2008a; Abbas et al., 2009; Mateo et al., 2017). Although in developed countries, the levels of aflatoxins present in foods and feed is regulated using quality standard technological tools, these regulations do not always apply to the farming done for food in rural communities in both under-developed and developing countries (Chackraborty and Newton, 2011). Some chemical control agents may be effective, however, there are harmful effects associated with their use. These include the health risks to those applying these control agents, and excess synthetic chemical fungicides draining off into the soil and contaminating ground water (Park et al., 1987; Sahab et al., 2014). Therefore, there is an increased demand for the investigation of alternative control agents, which are more natural, environmentally safer and biodegradable such as plant extracts.

The aim of this study was to provide insight into the antifungal and anti-aflatoxigenic properties of selected South African indigenous plants. This will provide the foundation towards understanding the mode of action of these plant extracts against aflatoxigenic-*Aspergillus* species. Furthermore, the cytotoxicity of these extracts was evaluated to determine their toxicity on two cell lines (HeLa and HepG2 cell lines).

3.2 Materials and Methods

3.2.1 Plant selection and collection of material

The selected plants namely, *Erythrophleum lasianthum*, *Heteropyxis natalensis* and *Warburgia salutaris*, were selected based on literature describing their traditional uses and for their reported antimicrobial properties (Gundidza et al., 1993; Monhanlall and Odhav, 2009; Nielsen et al., 2012). The leaves of the selected plants were collected from the Manie van Schijff Botanical Garden at the University of Pretoria, Pretoria,

South Africa in January 2018 (summer). Herbarium specimens of the three plants were prepared and submitted to the H.G.W.J. Schweickerdt Herbarium at the Department of Plant and Soil Science, University of Pretoria, for identification and assignment of voucher numbers (Table 3.1).

Table 3.1: The selected plants evaluated for their antifungal properties against *Aspergillus flavus* and *A. parasiticus*.

Plant name	Type of specimen	Voucher number*
E. lasianthum	Leaves	123561
H. natalensis	Leaves	124366
W. salutaris	Leaves	123558

*Voucher number: PRU number

3.2.2 Extraction

Plant material was collected and the leaves were air-dried. Thereafter, the dried leaves were ground to a fine powder using a leaf grinder (IKA mill continuous feeder-grinder, Cole-Parmer) and soaked in 500 ml of acetone, ethanol and water, and placed on a shaker for three days. The different solutions were then filtered under vacuum using Whatman No.1 filter paper. The filtrate of each sample was then concentrated by solvent evaporation in a fume hood. The dry extracts were then stored in polytops at ± 5 °C until use. The extracting solvents were chosen based on literature describing their ability to extract volatile and polar compounds miscible with polar and non-polar solvents (Eloff, 1998).

3.2.3 Fungal species

The fungal species used in this study were *Aspergillus flavus* (MRC 3951) and *A. parasiticus* (MRC 8986). These fungal species were obtained from the Agricultural Research Council (ARC), Plant Protection Research, Biosystematics Division, Roodeplaat, Pretoria, South Africa, where they were isolated from maize (*Zea mays*) and mopane worm (*Gonimbrasia belina*) debris, respectively. The two fungal species have been reported to be aflatoxin-producing isolates. However, AFB_1 detection using

Liquid chromatography tandem-Mass spectrometry (LC-MS/MS) at the Central Analytical Facility (CAF) at the University of Stellenbosch, Stellenbosch, South Africa, indicated that only the *A. flavus* isolate produced AFB_1 (333.63 ng/mL). These fungal species were routinely maintained by sub-culturing them onto Petri dishes containing either Potato Dextrose Agar (PDA) or Malt Extract Agar (MEA) then incubated at 25 ± 1 °C for 5 to 7 days. Fresh cultures were prepared 5 to 7 days before each experiment.

3.2.4 Bioassays

3.2.4.1 Fungal growth inhibition assay

A modified broth microdilution method as described by Masoko et al. (2005) was used to determine the minimum inhibitory concentration (MIC) values for each plant extract against *A*. *flavus* and *A. parasiticus*. This method is used to determine the MIC values, which constitute as a quantitative measure of the antimicrobial activity against bacteria or fungi (Balouiri et al., 2016).

Dried plant extracts (30 mg of each) were dissolved in 1 mL of 10 % DMSO (Dimethyl sulphoxide), to obtain stock concentrations of 30 mg/mL. One hundred microlitres (100 µL) of each plant extract was added into the first row of a 96-well microtiter plate containing 100 µL of Malt Extract Broth (MEB). Thereafter, a serial dilution of 100 µL from the wells of row A to B, B to C until H, was carried out. At the end of the serial dilution, in row H (1-12), 100 µL of the mixture was discarded. Therefore, each well contained 100 µL of the MEB and treatment sample (plant extract) mixture while the control wells contained 10 % DMSO (negative control) and amphotericin B (positive control) instead of the plant extracts. The wells used for the sterility control contained 200 µL of MEB only.

Using a 96-well plate reader, spore suspensions of *A. flavus* and *A. parasiticus* were prepared using sterile MEB. The spore suspension concentrations were adjusted to 0.5 Fungi McFarland standard (~1.5×10^8 cfu/mL). For each fungus, the spore suspension (100 mL) was transferred into the wells, except for the sterility control wells. The negative control, 10 % DMSO, was dissolved in 900 µL of distilled water, which together with the positive control, amphotericin B (250 µg/mL) were transferred into the

respective wells. The sterility control wells contained only 200 µL of MEB. The presence of fungi in the wells was indicated by 40 µL of 0.4 mg/mL of *p*-iodonitrotetrazolium chloride (INT) dissolved in 10 mL of distilled water. The indicator (INT) was added into all the wells. The microtiter plates were closed and sealed with parafilm, then incubated at 25 ±1 °C for 3 days. The MIC values were recorded as the lowest concentration at which the extracts and controls inhibited fungal growth. This was evident by lack of the pink colour in the wells, while the presence of fungal growth was indicated by a visible pink colour in the wells. The minimum fungicidal concentration (MFC) was determined by resuspending 50 µL from the wells, which did not show any fungal growth, into 150 µL freshly prepared broth (MEB). The resuspensions were incubated for 24 hours at 25 ±1 °C. The MFC values were determined as the lowest concentration at which the extract completely (100 %) inhibited fungal growth (modified from Mongalo et al., 2018). This experiment was carried out in triplicate and repeated three times.

3.2.4.2 Aflatoxin B_1 reduction assay on AFB_1-producing *Aspergillus flavus*

Using a modified method by Kumar et al. (2007) and Das et al. (2014), the effect of the selected plant extracts *E. lasianthum* (ethanol), *H. natalensis* (water) and *W. salutaris* (acetone) on the production of AFB_1 by *A. flavus* was evaluated. In conical flasks containing 50 mL Czapek Dox broth, 15, 30 and 75 mg/mL of each plant extract dissolved in 10 % DMSO, were mixed, respectively. The controls contained 10 % DMSO (negative control) and broth only (positive control). Each conical flask was then inoculated with 1 mL of 0.5 Fungi McFarland standard (~1.5 $\times 10^8$ cfu/mL) of *A. flavus* spore suspension, sealed with perforated parafilm and incubated at 28 ± 2 °C with agitation at 130 rpm for 15 days. After 15 days, the broth was filtered using Whatman No. 1 filter paper. The obtained wet and dry mycelial weight was recorded. The filtrate was collected and mixed with equal volumes (50 mL) of chloroform. The mixture was left on a shaker for 1 hour, after which the chloroform layer was collected using a separatory funnel. The chloroform layer was then air dried in a fume hood for 2 days. The dry residue was then re-dissolved in chloroform (1 mL). The re-dissolved solution was used for AFB_1 detection through spotting on Thin layer chromatography (TLC)

(silica gel) plates. The plates were then developed in chloroform: ethyl acetate (8:2) and acetone: chloroform (1:9) (Kumar et al., 2007).

Since, the fluorescing sample spots did not move up the TLC plate with the developing solvent [chloroform: acetone (9:1)], in an attempt to detect the presence of AFB_1 in the samples using the TLC method, various other solvent systems were used. The various solvent systems used to detect the presence of AFB_1 included benzene: acetonitrile (95:5), acetone: water (60:40), methanol: water (7:3) and chloroform: acetone (9:1), chloroform: acetone (96:4), ether: methanol: water (96:3:1), water: acetonitrile (9:1) and methanol: water (80:20) v/v (Das et al., 2014; Abhishek et al., 2015; Mateo et al., 2017; Sun and Zhao, 2018).

The AFB_1 content in the samples was quantitatively estimated by scraping the fluorescing spots on the TLC plates and dissolving the sample scrapings in cold methanol (5 ml). The methanol solution containing the scraped spots were shaken and centrifuged at 3000 rpm for 5 minutes. The spectrophotometer (Du 720 Beckman Coulter, Midrand, South Africa) was used to determine the absorbance of the supernatant at a wavelength of 360 nm. The amount of AFB_1 and AFB_1 reduction in each sample was then calculated using the following equations (Kumar et al., 2007; Abhishek et al., 2015), respectively:

$$AFB1\ content\ \left(\frac{\mu g}{L}\right) = \frac{D \times M}{E \times L} \times 1000$$

where D = absorbance, M = molecular weight of AFB_1 (312 g/mol), E = molar extinction coefficient of AFB_1 (21 800) and L = path length (1 cm).

$$\%\ \mathrm{AFB1}\ \ \mathrm{reduction} = \frac{1 - \mathrm{T}}{\mathrm{C}} \times 100$$

where T = AFB_1 concentration in the treatment sample (µg/l), C = AFB_1 concentration in positive control (µg/L).

The procedure was repeated as two independent experiments, in triplicate.

One milligram of each sample and 50 (µg/ml) AFB_1 standard were also submitted to the Central Analytical Facility (CAF) at the University of Stellenbosch, Stellenbosch, South Africa, for AFB_1 analysis using Liquid chromatography tandem-Mass spectrometry (LC-MS/MS).

3.2.4.3 Cytotoxicity of the plant extracts

The five extracts (*E. lasianthum* ethanol, *H. natalensis* acetone, ethanol, water and *W. salutaris* acetone) which exhibited noteworthy (MIC ≤ 1 mg/mL) antifungal activity against *A. flavus* and *A. parasiticus* were further evaluated for their toxic effect on epithelial cell substitutes (HeLa- Human cervix epithelial carcinoma cells) and liver cell substitutes (HepG2- Malignant human hepatoma cells). The cytotoxicity of the plant extracts was determined using the microtiter PrestoBlue assay (MPSA) (Lall et al., 2013).

The HeLa cells were grown in minimum essential medium (MEM) whilst the HepG2 cells were grown in Dulbecco's modified eagle medium (DMEM) supplemented with 10 % Fetal calf serum and 1 % antibiotics (5 mL mixture of Penicillin, Amphotericin B and Streptomycin) at 37 °C with 97 % humidity and a 5 % CO_2 atmosphere. A hundred microliters of cells (10 000 cells per well) were then subcultured into 96-well microtiter plates by treating them with trypsin-EDTA (Ethylene diamine tetra-acetic acid) for 10 minutes and incubating them for 24 hours to allow them to attach to the bottom of the microtiter wells. The cells were then treated with serially diluted 2 mg/mL plant extracts (3.1- 400 µg/mL) and Actinomycin D (3.91×10^{-6} - 0.05 µg/mL) and further incubated for 72 hours. After the 72-hour incubation period, 20 µL of PrestoBlue indicator was added to each well and incubated for two hours. The colour change was then observed and used to determine the viability of the cells in each microtiter well. The results were spectrophotometrically quantified at 600 nm (reference wavelength) and at 570 nm, using an ELISA (enzyme-linked immunoassay) plate reader (BIO-TEK Power-Wave XS, Weltevreden Park, South Africa). The obtained absorbance values were further analyzed using the Graphpad Prism 4 statistical analysis software, to determine the fifty percent inhibitory concentration (IC_{50}) values for each extract. The IC_{50} values in mg/mL

were then used to calculate the selectivity indices of each extract on each cell line using the following equation:

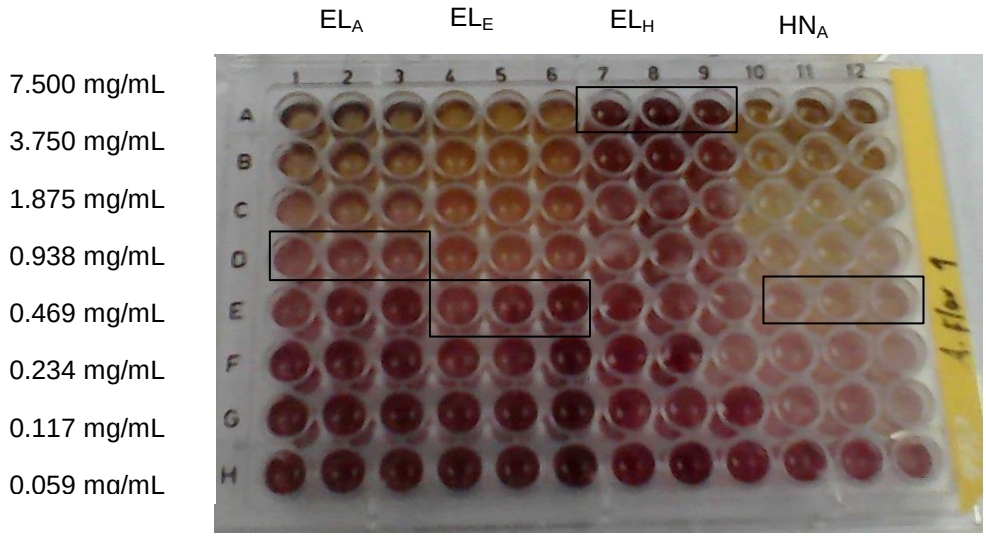
$$\text{Selectivity index (SI)} = \frac{\text{IC50}}{\text{MIC}} \text{ in mg/mL}$$

3.3 Results and Discussion

Limited research has been done on the antifungal activity of *E. lasianthum*, *H. natalensis* and *W. salutaris* extracts against *Aspergillus* species. Therefore, this is the first report of the selected plant extracts, with the exception of *W. salutaris* acetone, against the growth of *A. flavus* and *A. parasiticus in vitro* and as AFB_1 reducers.

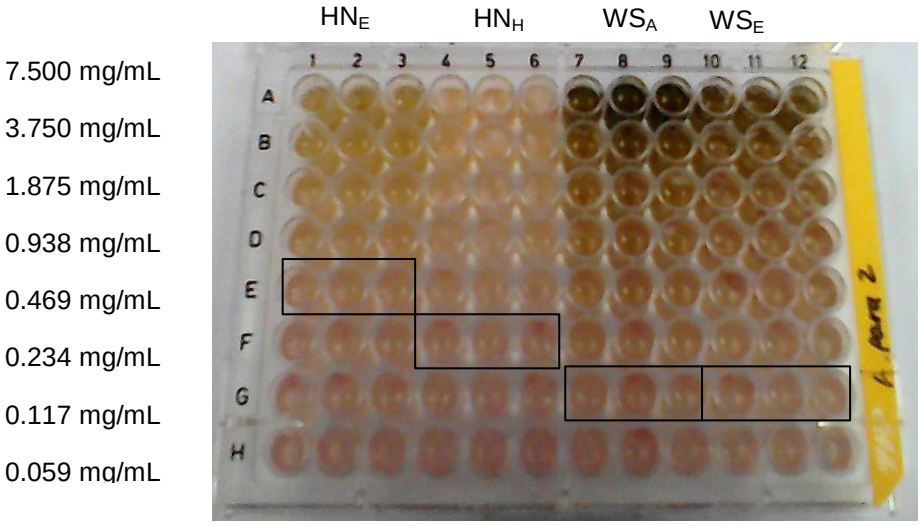
3.3.1 Fungal growth inhibition of *A. flavus* and *A. parasiticus*

The antifungal potential of the plant extracts was indicated by a lack or presence of the pink colour in the wells (Figure 3.1 and 3.2). The presence of the pink colour indicated fungal growth, while a lack of the pink colour indicated inhibition of fungal growth. Furthermore, the average minimum inhibitory concentration (MIC) values were calculated and recorded.



* EL$_A$: *E. lasianthum* acetone, EL$_E$: *E. lasianthum* ethanol, EL$_H$: *E. lasianthum* water, HN$_A$: *H. natalensis* acetone,

Figure 3.1: Antifungal screening of the selected plant extracts against *A. flavus* growth using the broth microdilution method and *p*-iodonitrotetrazolium chloride (INT) as a growth indicator. The minimum inhibitory concentration (MIC) is indicated by the rectangular blocks.



*HN$_E$: *H. natalensis* ethanol, HN$_H$: *H. natalensis* water, WS$_A$: *W. salutaris* acetone, WS$_E$: *W. salutaris* ethanol

Figure 3.2: Antifungal screening of the selected plant extracts against *A. parasiticus* growth using the broth microdilution method and *p*-iodonitrotetrazolium chloride (INT) as a growth indicator. The minimum inhibitory concentration (MIC) is indicated by the rectangular blocks.

Minimum inhibitory concentration values less than 8 mg/mL are considered to have antimicrobial activity (Rios and Recio, 2005). However, MIC values less than 1 mg/mL are considered to have noteworthy antimicrobial activity (Rios and Recio, 2005; Van Vuuren, 2008). The average MIC values for the different plant extracts against *A. flavus* and *A. parasiticus* are indicated in Table 3.2. The initial concentration of each plant extract in the different wells was 7.5 mg/mL. The three different plants extracted with acetone, ethanol and water all showed moderate (< 8.0 mg/mL) antifungal activity against both *A. flavus* and *A. parasiticus* (Table 3.2). However, the extracts which showed noteworthy (≤ 1 mg/mL) antifungal activity against *A. flavus* were *E. lasianthum* (ethanol), *H. natalensis* (acetone, ethanol, water) and *W. salutaris* (acetone, ethanol). Similarly, *H. natalensis* (acetone, ethanol, water) and *W. salutaris* (acetone) showed noteworthy (≤ 1 mg/mL) antifungal activity against *A. parasiticus*.

Table 3.2: The average minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) values for the different selected plant extracts against *A. flavus* and *A. parasiticus*.

	A. flavus		***A. parasiticus***	
Extract	**MIC (mg/mL)**	**MFC (mg/mL)**	**MIC (mg/mL)**	**MFC (mg/mL)**
E. lasianthum A	1.875	1.875	1.875	1.875
E. lasianthum E	**0.234**	**0.469**	1.875	3.750
E. lasianthum H	NA	NA	NA	NA
H. natalensis A	**0.469**	**0.469**	**0.469**	**0.469**
H. natalensis E	**0.938**	**0.938**	**0.938**	**0.938**
H. natalensis H	**0.938**	1.875	**0.469**	**0.938**
W. salutaris A	**0.117**	**0.234**	**0.117**	**0.234**
W. salutaris E	**0.938**	**0.938**	1.875	1.875
W. salutaris H	NA	NA	NA	NA
Amphotericin B (positive control)	0.125	0.125	0.063	0.063

*A: acetone; E: ethanol; H: water, NA: not active and bold values: MIC values (≤ 1 mg/mL).

There were differences in the MIC values noted for each plant extract against *A. flavus* and *A. parasiticus* (Table 3.2). Interestingly, *H. natalensis* showed overall the best antifungal activity against *A. parasiticus*. In contrast, Gundidza et al. (1993) observed, using the poisoned food method, that *H. natalensis* essential oils were able to inhibit the fungal growth of *A. flavus* and *A. parasiticus* by 99.2 and 97.3 %, respectively, therefore indicating that the *H. natalensis* essential oils had better antifungal activity against *A. flavus* when compared to their effect on *A. parasiticus*. The ethanolic extract of *E. lasianthum* (0.234 mg/mL) and the acetone extract of *W. salutaris* (0.117 mg/mL) showed better antifungal activity against *A. flavus* when compared to the ethanolic

(0.938 mg/mL) and acetone (0.469 mg/mL) extracts of *H. natalensis*. Among, the water extracts, *H. natalensis* (0.938 mg/mL) showed the best antifungal activity compared to the other water plant extracts (7.500 mg/mL).

Additionally, the water extracts of *W. salutaris* and *E. lasianthum* showed the least antifungal activity against both *A. flavus* and *A. parasiticus* but this result may have been due to water as an extractant not being able to extract compounds responsible for the antifungal activity against *A. flavus* and *A. parasiticus*.

The lowest MIC value obtained for the antifungal activity against *A. flavus* was from the *W. salutaris* (acetone) extract with an MIC value of 0.117 mg/mL. In contrast, Dikhoba et al. (2019) reported that the acetone extract of *W. salutaris* extract, against *A. flavus*, had MIC values of 0.780 mg/mL after 24 and 48 hour incubation periods. This is relatively 6-fold higher compared to the MIC value (0.117 mg/mL) obtained after the 73 hour incubation period carried out in this study. Similar to Dikhoba et al.'s (2019) study, Mongalo et al. (2018) reported that the water and methanol: dichloromethane (1:1) extracts of *W. salutaris* against *A. flavus*, had MIC values as low as 0.780 mg/mL and 0.160 mg/mL, respectively. However, the methanol: dichloromethane (1:1) extract was reported to have a 0.020 mg/mL MIC value against *A. parasiticus*, which is lower than what was obtained against *A. flavus* (Mongalo et al., 2018). In this study, the opposite was noted for the acetone extract of *W. salutaris*, as the antifungal activity against *A. flavus* was indicated by the same MIC value obtained against *A. parasiticus* (0.117 mg/mL). However, extracts of *H. natalensis* (acetone, water), *E. lasianthum* (acetone, water) and *W. salutaris* (water) showed the same activity against *A. flavus* and *A. parasiticus*. In comparison, Gomez et al. (2019) noted that extracts of plants from northwest Argentina had lower MIC values for the antifungal activity against *A. parasiticus* (MIC= 188 µg/mL) than for *A. flavus* (MIC= 750 µg/mL). In contrast, Moghadam et al. (2016) reported that the MIC values of *Ziziphora clinopodioides* were the same (48.82 µg/mL) against both *A. flavus* and *A. parasiticus*.

The extracts which showed noteworthy (≤ 1 mg/mL) MFC against *A. flavus* were *E. lasianthum* (ethanol), *H. natalensis* (acetone and ethanol) and *W. salutaris* (acetone and ethanol), with MFC values of 0.469, 0.469, 0.938, 0.234 and 0.938 mg/mL,

respectively. In contrast, the extracts which exhibited noteworthy (≤ 1 mg/mL) activity against *A. parasiticus* were *H. natalensis* (acetone, ethanol and water) and *W. salutaris* (acetone) with MFC values of 0.469, 0.938, 0.234 and 0.938 mg/mL, respectively.

In this study, the different plant extracts showed variations in the antifungal activities against *A. flavus* and *A. parasiticus* growth *in vitro*. Most likely, the antifungal potential or activity of an extract is not only dependent on the concentration of the extract. It may also be dependent on the type of compounds present in the extract, which may be the main contributors towards the extract having a lesser/greater mode of antifungal activity compared to other plant extracts. This is because the type of compounds in any plant extract play an important role in their mode of action. In addition, the solvent used during extraction may also have influenced the extracts' ability to inhibit fungal growth, as some extractants are able to extract the most polar or more non-polar compounds present in plants (Cowan, 1999).

3.3.3 Aflatoxin B_1 reduction of AFB_1-producing *Aspergillus flavus*

The effect of the selected plant extracts, *E. lasianthum* (ethanol), *H. natalensis* (water) and *W. salutaris* (acetone) on mycelial growth and the production of AFB_1 were recorded and compared to the positive control. In this study, AFB_1-producing *A. flavus* in liquid culture treated with different plant extract concentrations, produced less AFB_1 than the untreated cultures. A maximum of 49 % AFB_1 reduction was noted at 75 mg/mL of *W. salutaris* (acetone), when compared to the lowest tested concentration of 15 mg/mL *W. salutaris* (acetone), where a maximum of 30 % AFB_1 reduction was noted (Table 3.3). Similarly, Gali et al. (2010) reported that aqueous extracts of *Terminalia chebula* were able to reduce AFB_1 production by *A. flavus* and *A. parasiticus* by 45 and 54 % at a tested concentration of 25 %. Mohseni et al. (2014) noted that aqueous extracts of *Glycyrrhiza glabra* were able to inhibit AFB_1 production by *A. parasiticus* at extract concentrations of 10 mg/mL.

In this study, a general trend was observed with the reduction of AFB_1 produced by extract treated *A. flavus*. As the concentration of the extract increased, there was, a decrease in the production of AFB_1, considering the plant extract had aflatoxin reducing

properties (Table 3.3). Therefore, the reduction of AFB_1 by plant extract treated *A. flavus* was partially dependent on the concentration of the plant extract when compared to the untreated cultures. Similar to observations in this study, Sanchez et al. (2005) observed that the methanolic extract of *Agave asperrima* at 0.125, 0.250 and 0.325 mg/mL extract concentrations were able to reduce AFB_1 production by 16, 65 and 90 %, respectively.

In this study, no significant reduction in dry mass of mycelia was observed with all the tested concentrations of the extract (Appendix A, Table 8.1). In contrast, Abdel-Fattah et al. (2018) using the Agar-well disk diffusion assay and Mueller Hinton Agar noted that there was a reduction in the mycelial growth and AFB_1 produced as the plant extract concentration (Wild *Stevia*, 10 mg/mL) increased. Furthermore, Namazi et al. (2002) and Abdel-Fattah et al. (2018) postulated that this observation may be due to fungicidal compounds present in the extracts, which have the ability to lyse the cell membranes of mycelia and spores, thus resulting in decreased fungal growth. However, since no reduction in fungal growth was observed in this study, the mode of antifungal action of the tested extracts may be different from the mode of action postulated by Namazi et al. (2002) and Abdel-Fattah et al. (2018).

Table 3.3: The aflatoxin B_1 (AFB_1) reduction percentages (%) as a result of treating AFB_1-producing *A. flavus* with selected plant extracts.

Treatment	AFB_1 content (µg/L)	AFB_1 reduction (%)
Standard	1.095	-7.3
E. lasianthum 15	0.890	12.82
E. lasianthum 30	0.820	15.66
E. lasianthum 75	0.790	26.59
H. natalensis 15	0.720	26.38
H. natalensis 30	0.770	33.93
H. natalensis 75	0.690	42.82
W. salutaris 15	0.670	**30.53**
W. salutaris 30	0.660	**46.40**
W. salutaris 75	0.640	**49.27**
Positive control	1.295	-
Negative control	1.170	-13.13

*15: 15 mg/mL; 30: 30 mg/mL; 75: 75 mg/mL extract concentrations and bold values indicate the best AFB_1 reduction for each extract concentration.

The results from the analysis of AFB_1 using LC-MS/MS indicated that AFB_1 was only present in the positive control (332.63 ng/mL) and not in any of the other samples (treated and the negative control). There is a possibility that the sensitivity of the LC-MS/MS system could not detect the minute AFB_1 concentrations in the other samples (negative control and treated) except in the positive control.

However, the extracts could have degraded AFB_1 into different products and that the presence of 10 % DMSO in the negative control may have contributed to the formation of AFB_1 derivatives, which could not be detected using LC-MS/MS. Similarly, Iram et al. (2016) reported that the detoxification of AFB_1 using aqueous plant extracts can often lead to its degradation. Furthermore, the degraded products are often due to the removal of the double bond present in the terminal furan ring or modification to the lactone group of AFB_1 (Iram et al., 2016). These changes in the chemical composition of AFB_1 result in the modified product being less toxic because the double bond present in the furan ring is the main contributor responsible for the toxic and carcinogenic properties of AFB_1 (Wang et al., 2011; Iram et al., 2016).

3.3.4 Cytotoxicity

The extracts, which showed noteworthy (MIC ≤ 1 mg/mL) antifungal activity, were selected for cytotoxicity evaluation (Chapter 3.4.1). The extracts chosen were *E. lasianthum* (ethanol), *H. natalensis* (acetone, ethanol and water) and *W. salutaris* (acetone) (Figure 3.3, Table 3.4).

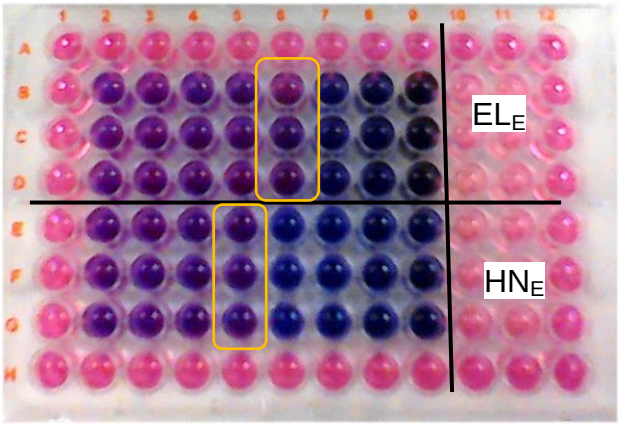


Figure 3.3: Cytotoxicity of the ethanolic extracts of *E. lasianthum* and *H. natalensis* on the HeLa cell line. The 50 % inhibitory concentration (IC_{50}) is represented as the colour change from blue to purple (shown in the orange shapes).

The cytotoxicity of the selected plant extracts on the two cell lines (HeLa and HepG2) were determined in comparison to the cells grown in medium (MEM and DMEM) only

(negative control) and cells treated with Actinomycin D (positive control). The basic concept of fifty percent inhibitory concentration (IC_{50}) values are that high IC_{50} values imply that a larger quantity of the extract is required to cause toxicity. In contrast, lower IC_{50} values imply that small quantities of the extract are required to cause toxicity and could therefore cause harm at low concentrations (Okeleye et al., 2013).

Table 3.4: The cytotoxicity of the selected plant extracts on HeLa and HepG2 cell lines and their selectivity indices, indicated as IC_{50} values.

	Plant extracts				
	E. lasianthum E	*H. natalensis* A	*H. natalensis* E	*H. natalensis* H	*W. salutaris* A
HeLa (µg/mL)	**106.39**	**134.73**	**116.48**	73.25	96.01
HepG2 (µg/mL)	**153.20**	87.15	**>400.00**	22.12	38.86
SI value per fungal strain (HeLa cell line)					
A. flavus	0.455	0.286	0.124	0.078	0.821
A. parasiticus	0.057	0.287	0.124	0.156	0.102
SI value per fungal strain (HepG2 cell line)					
A. flavus	0.654	0.186	0.426	0.024	0.332
A. parasiticus	0.082	0.186	0.426	0.047	0.041

*A: acetone; E: ethanol; H: water extracts and bold values: $IC_{50} \geq 100$ µg/mL.

The IC_{50} values of the tested extracts were found to range between 22.12 and greater than 400 µg/mL (Table 3.4). The extracts tested on the HepG2 cell line indicated a decrease in cell viability as the concentration of the extracts increased (Figure 8.2, Appendix A). Additionally, the water extract of *H. natalensis* and the acetone extract of *W. salutaris*, which showed moderate toxicity (50 µg/mL ≥ IC_{50} ≤ 100 µg/mL) on the HeLa cell line (Figure 8.1, Appendix A) were toxic (IC_{50} < 50 µg/mL) to the HepG2 cell line. The ethanolic extracts of *E. lasianthum* and *H. natalensis* showed no toxicity (IC_{50} > 100 µg/mL) at the highest tested (400 µg/mL) concentration on both the HeLa and the HepG2 cell lines (Figure 3.3, Table 3.4).

Previous studies have tested the plant extracts of the selected plants (*E. lasianthum*, *H. natalensis* and *W. salutaris*) on HeLa (human cervical carcinoma), HepG2 (malignant human hepatoma), Vero (monkey kidney) and RAW (mouse macrophage) cell lines (Madikane et al., 2007; Twilley et al., 2017; Mongalo et al., 2018; Soyingbe et al., 2018). The ethanolic leaf extracts of *E. lasianthum* and *W. salutaris* on the HeLa cell line were reported by Twilley et al. (2017) to have IC_{50} values greater than 200 µg/mL for both extracts. However, the methanol, ethyl acetate, acetone and water extracts of *W. salutaris* on the HeLa cell line exhibited IC_{50} values of 84.00 (±1.25), 71.27 (±0.09), 56.01 (±0.38) and 77.50 (±0.11) µg/mL, respectively (Soyingbe et al., 2018). Dzoyem et al. (2015) also pointed out that the acetone extracts of *H. natalensis* and *W. salutaris* on the Vero cell line had IC_{50} values of 264.05 and 74.64 µg/mL, respectively. Interestingly, the susceptibility of the HepG2 cell line to the acetone extracts of *H. natalensis* and *W. salutaris* was higher compared to the HeLa cell line, whose IC_{50} values indicated similar cell toxicity to the toxicity observed by Dzoyem et al. (2015) on the Vero cell line. The moderate toxicity of the acetone extract of *W. salutaris* on the HeLa cell line (96.01 µg/mL) is similar to that reported by Soyingbe et al. (2018) (56.01 µg/mL) and similar to the toxicity reported by Dzoyem et al. (2015) on the Vero cell line (74.64 µg/mL).

In this study, the selectivity indices (SI) for the selected plant extracts using the MIC values obtained against *A. flavus* and *A. parasiticus* ranged between 0.024 and 0.821 (Table 3.4). The selectivity index of an extract, which is determined by the ratio of the toxicity of the extract on cells and its activity on a pathogen, indicates the extracts' relative safety (Makhafola et al., 2014). Therefore, the higher the SI value, the greater the safety margin between the concentration required, in this case, for fungicidal activity and the concentration at which the extract is toxic to the cell line used (Makhafola et al., 2014). The lowest SI value (0.024) was noted for the water extract of *H. natalensis* against *A. flavus* tested on the HepG2 cell line, while the highest SI value was from the acetone extract of *W. salutaris*, which interestingly showed moderated toxicity on the HeLa cell line.

Therefore, the interpretation of the SI values *in vivo*, as indicated by Elisha et al. (2017) that an SI value below 1 implies that the extract has greater toxicity against the pathogen than it has on the cells. Although, in this study all the obtained SI values were below 1, the activity of the selected extracts can be explained using Elisha et al.'s (2017) statement that the toxicity of extracts, once administered to humans and animals, may actually exhibit different (pharmacodynamic and pharmacokinetic) effects when compared to the efficacy observed *in vitro*.

3.4 Conclusion

The antifungal screening of the three plant extracts extracted with acetone, ethanol and water, confirmed that these extracts are able to inhibit the growth of *A. flavus* and *A. parasiticus in vitro*. The three most active plant extracts (*E. lasianthum* ethanol, *H. natalensis* water and *W. salutaris* acetone) identified from the antifungal screening also reduced the production of AFB_1 in broth after a 15-day incubation period when compared to the untreated sample containing broth and the fungus only. The same extracts indicated that at the highest tested concentration (400 µg/mL), only *H. natalensis* (water) and *W. salutaris* (acetone) were toxic to liver cell substitutes (HepG2 cells) when compared to the other tested extracts. Therefore, since the selected plant extracts have the potential to inhibit *A. flavus* and *A. parasiticus* growth, optimization of the concentrations of the selected plant extract could lead to the formulation of a natural biocontrol agent specific against *A. flavus* and *A. parasiticus*. Furthermore, these plant extracts can be further studied for integration in pest management of other *Aspergillus* species and other phytopathogenic fungi.

Therefore, future research may consider evaluation higher concentrations of the plant extracts in order to determine the concentration at which these extracts are able to produce optimum fungicidal effect against *A. flavus* and *A. parasiticus*. In addition, future research into identification of the active anti-*Aspergillus* and anti-aflatoxigenic compounds in the extracts and their mode of action, which contributes to their fungistatic and fungicidal effects on *A. flavus*, *A. parasiticus* and AFB_1 production, may be necessary.

Chapter 4
Isolation and identification of potentially active compounds from *Heteropyxis natalensis*

4.1 Introduction

The continuous use of plants worldwide, as treatment for diseases and against pathogens has increased the discovery of drug novelties from plants through research into the compounds present in plants (Phillipson, 2001). This is due to high-throughput screening bioassays and tests, which indicate bioactivity and often lead to the isolation of active compounds (also known as bioassay-guided fractionation) (Phillipson, 2001). In some instances, the use of plant extracts as a whole, have been replaced by single chemical entities isolated from plants (Phillipson, 2001). One of the main advantages of isolating compounds from natural products (such as plant extracts, which are often feasible screening material) generates naturally derived compounds with either improved or better efficacy and whose activity is sometimes unrelated to its known screening biological function (Galloway et al., 2009; DeCorte, 2016; Khan, 2018). Moreover, it is a crucial step towards standardisation for production formulation and development (Lorenz et al., 2017). Another advantage of isolation is the removal of toxic compounds, as mixtures are more likely to contain toxic constituents (Cowan, 1999).

Chemical constituents produced by plants are categorised into two groups, namely primary and secondary metabolites (Ruchika et al., 2019). Primary metabolites are naturally produced by plants and are essential for plant growth and development (Van Vuuren et al., 2007; Ruchika et al., 2019). In contrast, secondary metabolites are produced in response to pathogens and other external factors or stressors such as climate and the quality of the soil (Van Vuuren et al., 2007; Ruchika et al., 2019). Therefore, due to the influence of external factors, the chemical composition of a plant may vary quantitatively and qualitatively (Van Vuuren et al., 2007). Furthermore, since

the biological activity of plant extracts is due to its chemical composition, its activity is bound to vary as a consequence of the effect of the external factors on the production of phytochemicals (Van Vuuren et al., 2007).

Heteropyxis natalensis Harvey, also known as the lavender tree, is a deciduous tree, which has been used, in traditional medicine (Hutchings et al., 1996; Van Wyk et al., 1997). Its antimicrobial activity has also been evaluated against oral pathogens, *Cryptococcus neoformans*, *Aspergillus flavus*, *A. niger*, *A. ochraceus*, *A. parasiticus* and Gram-positive and Gram-negative bacteria (Gundidza et al., 1993; Henley-Smith et al., 2018). Although the phytochemistry of *H. natalensis* has been studied, fewer compounds have been isolated from the leaf extracts. One of the major groups of compounds that were reported to be present in *H. natalensis* leaf extracts were the monoterpenoid group of compounds (Van Wyk et al., 1997). The monoterpenoid compounds isolated from *H. natalensis* were reported to be mainly *β*-ocimene, 1, 8 cineole, limonene, linalool and myrcene (Van Wyk et al., 1997).

Chemical constituents from plant extracts and essential oils have been used as alternative fungal growth control and reducing agents (Tian et al., 2018). Various monoterpenoid compounds which have been isolated from plant essential oils and extracts include compounds such as thymol, camphor, borneol, carvacrol, bornylacetate and *p*-cymene [1-methyl-4-(1-methylethyl)-benzene] (Lis-Balchin et al., 1998; Paranagama et al., 2003; Rojas-Grau et al., 2007). These chemical constituents have been reported to have antifungal properties against *Aspergillus* species, however, knowledge on their mode of action is very limited (Lis-Balchin et al., 1998; Paranagama et al., 2003; Rojas-Grau et al., 2007).

Therefore, the demand for use of natural and environmentally sustainable products against fungal spoilage and the production of mycotoxins, especially in storage, is increasing as a result of the many side effects associated with the use of synthetic fungicides (Sahab et al., 2014). Plant extracts and plant-based products, which are biodegradable and have minimum to no residual toxicity, are being investigated as control agents against phytopathogenic fungi (Salehan et al., 2013). Furthermore, the presence of different biologically active compounds produced by plants such as tannins,

alkaloids and flavonoids, increases the potential for plant extracts to reduce and/or inhibit fungal contamination in the field and/or during storage (Soylu et al., 2006).

Moreover, plant extracts with antifungal activity are often studied further to determine the phytochemicals responsible for the activity. It is however, often challenging to link or correlate the mode of action and/or if there are specific classes of compounds present in the plant extract responsible for the antifungal activity observed (Da Cruz Cabral et al., 2013). This is due to the abundance of compounds that are found in plant extracts (Da Cruz Cabral et al., 2013). Therefore, the source of antifungal activity in plant extracts is often not established as a single compound to a one target cell because of the variety of compounds present in plant extracts (Da Cruz Cabral et al., 2013). In some instances, the many compounds have synergistic effects as antifungals when in combination in the plant extract than as single isolated entities (Da Cruz Cabral et al., 2013).

The broth microdilution bioassay results (Chapter 3 section 3.3.1) indicated that the leaf extracts of *H. natalensis* had noteworthy (≤ 1 mg/mL) activity against both *A. flavus* and *A. parasiticus*. Additionally, the ethanolic leaf extract of *H. natalensis* exhibited the least toxicity on HeLa and HepG2 cell lines (> 100 µg/mL) (Chapter 3 section 3.3.3). Therefore, based on the plant conservation status of the selected plants and the antifungal and cytotoxicity results, *H. natalensis* was chosen for fractionation and isolation of possible compounds responsible for the antifungal activity against *A. flavus* and *A. parasiticus* (Chapter 3).

4.2 Materials and Methods

4.2.1 Reagents

All the reagents and Thin layer chromatography (TLC) plates used, unless otherwise stated, were purchased from Sigma-Aldrich/Merck (Johannesburg, South Africa).

4.2.2 *Heteropyxis natalensis*: Collection and extraction

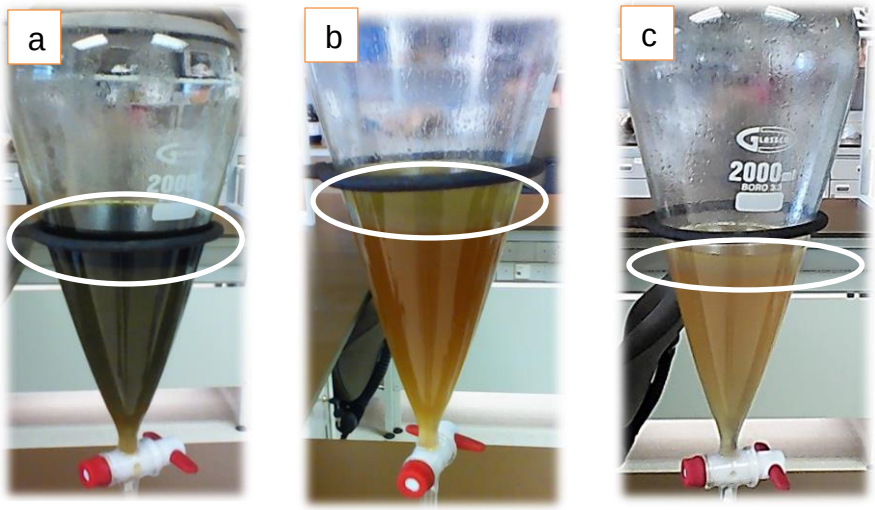
The leaves (collected from the same tree as mentioned in Chapter 3 section 3.2.1) were dried and ground to a fine powder using a leaf grinder (IKA mills MF 10.1 basic

continuous feed grinder, Cole-Parmer). The leaf powder was extracted twice using 500 ml ethanol for three days each time. The extract was filtered under vacuum using Whatman No.1 filter paper. The filtrate was then concentrated using a rotary evaporator (Buchi R-210 model, Labotec, South Africa) and evaporated in a fume hood cabinet. Once dry, the extract was stored in a polytop at ± 5 °C until use.

4.2.3 Liquid-liquid extraction of *H. natalensis*

The most convenient solvent system for the separation of compounds present in the *H. natalensis* extract was determined by dissolving 2 mg (*H. natalensis*) in ethanol. The dissolved extract was then spotted onto a TLC plate and run using the following mobile phases: ethyl acetate: methanol: water (6.5:2.0:1.0), *n*-hexane: ethyl acetate (7:3), ethyl acetate: methanol (99:1) to obtain TLC profiles of the extract.

The ethanolic *H. natalensis* extract (22 g) was then subjected to liquid-liquid partitioning or extraction. The extract was suspended in 500 ml of distilled water and transferred into a separatory funnel (2 L). The suspension was successively extracted using 250 mL of each of the following solvents: *n*-hexane, ethyl acetate, *n*-butanol and water (repeated four times per solvent) (Figure 4.1). The liquid-liquid partitioned extracts obtained were concentrated using a rotary evaporator, and then dried in a fume hood cabinet.



*The white oval shapes indicate the organic layers of each liquid-liquid partition step.

Figure 4.1: Liquid-liquid partitioning of the ethanolic extract of *Heteropyxis natalensis* using a) *n*-hexane b) ethyl acetate and c) *n*-butanol and water.

Once dry, the extracts were developed on a TLC plate to obtain the TLC profiles of each liquid-liquid partitioned extract in comparison to the ethanolic extract of *H. natalensis* (Figure 4.2). The extracts were also tested for their antifungal activity against both *A. flavus* and *A. parasiticus* using the broth microdilution method as described in Chapter 3 section 3.2.4.1 (further elaborated on in section 4.2.5). The minimum inhibitory concentration (MIC) values obtained (Table 4.1) were used to determine the liquid-liquid partitioned extract to be used for column chromatography.

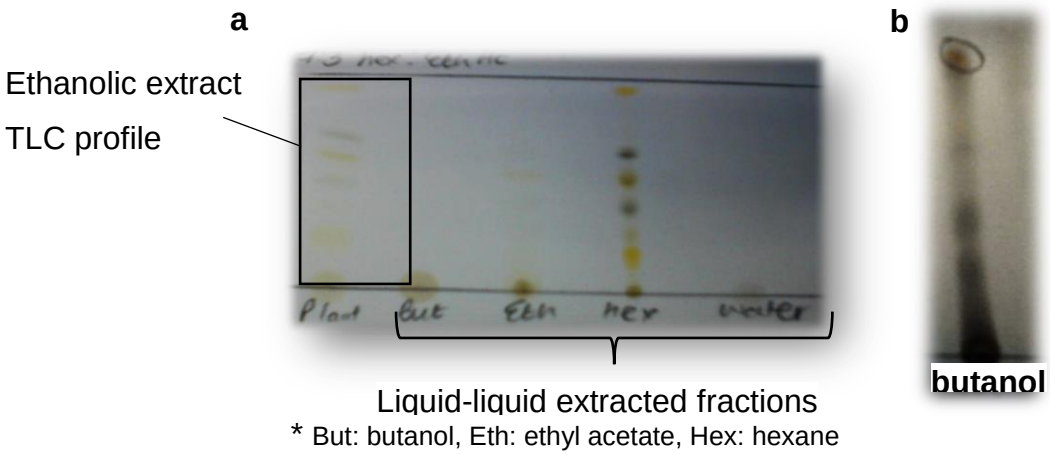


Figure 4.2: a) A TLC profile of the ethanolic extract of *H. natalensis* in comparison to the liquid-liquid extracted fractions developed using hexane: ethyl acetate (7:3) and b) The TLC profile of the butanol liquid-liquid partitioned extract, using the solvent system, ethyl acetate: methanol: water (6.5:2:1).

4.2.4 Isolation and fractionation of *H. natalensis* compounds

The butanol liquid-liquid partitioned extract (4.4 g), which showed the best antifungal activity, was then subjected to isolation and fractionation using column chromatography on a silica gel column (Figure 4.3).

Figure 4.3: Isolation of compounds from the *H. natalensis* extract using the butanol liquid-liquid partitioned fraction and silica gel column chromatography.

The glass column (8 m x 8 cm) was packed with silica gel (120 g) suspended in *n*-hexane. The butanol extract (4.4 g) and 12 g of silica was suspended in 4 mL methanol. The butanol extract and silica mixture was air dried for two hours in the fume hood cabinet and then loaded at the top of the silica packed column. Compound isolation was then eluted using mixed ratios of *n*-hexane, ethyl acetate and methanol, according to the increasing polarity of the fractions collected. In total, 286 fractions were collected; however, fractions containing similar compounds (according to the TLC profiles) were combined to give a total of 25 major pooled fractions. The TLC profiles were determined by spotting the fractions on TLC plates then developed using different solvent mixtures of *n*-hexane, ethyl acetate, methanol and water (Figure 4.4). After development of the TLC profiles, the TLC plates were dipped in acidic vanillin (0.34 % vanillin in 3.5 % sulphuric acid in ethanol) and heated to reveal compounds, which did not absorb UV light.

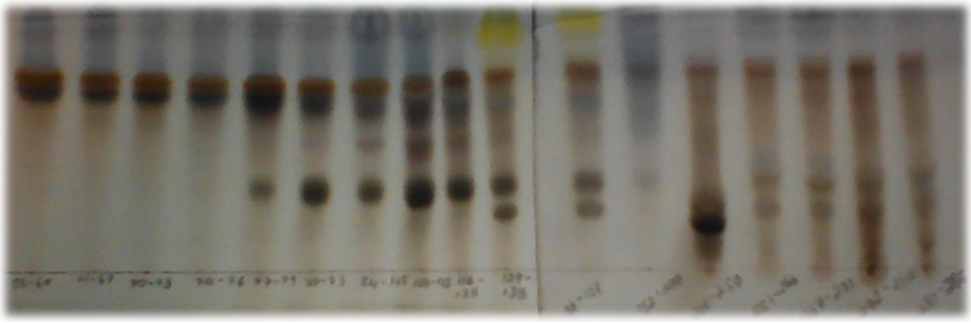


Figure 4.4: A TLC profile of the pooled fractions (58-286) of the *H. natalensis* extract using the butanol liquid partitioned fraction developed using ethyl acetate: methanol: water (6.5:2:1).

4.2.5 Antifungal activity of the column chromatography fractions from the butanol fraction of *H. natalensis* against *A. flavus* and *A. parasiticus*

The modified-broth microdilution method (Chapter 3 section 3.2.4) (Masoko et al., 2005) was used to determine the antifungal activity of the liquid-liquid partitioned fractions and the major isolated pooled fractions which were obtained through column chromatography. However, the only difference was the concentration of the stock solution of each extract, of which for this investigation, ranged between 1 and

30 mg/mL. The active pooled fraction was subsequently submitted for compound identification using the NIST library database and GC-MS (Appendix A, Chapter 8 section 8.1) at the Department of Chemistry, University of Pretoria, Pretoria, South Africa (Table 4.3).

4.3 Results and Discussion

4.3.1 Antifungal activity of the *H. natalensis* liquid-liquid partitioned fractions against *A. flavus* and *A. parasiticus*

The fractions obtained from liquid-liquid partitioning of the ethanol extract of *H. natalensis* showed good (< 8 mg/mL) (Rios and Recio, 2005; Van Vuuren, 2008) antifungal growth inhibition against both *A. flavus* and *A. parasiticus* (Table 4.1).

Table 4.1: The antifungal activity of the liquid-liquid partitioned *H. natalensis* fractions against *A. flavus* and *A. parasiticus* growth *in vitro* using the broth microdilution method.

	MIC (mg/mL)	
Liquid-liquid partitioned fraction	*A. flavus*	*A. parasiticus*
n-Butanol	**0.469**	0.469
Ethyl acetate	0.938	**0.234**
n-Hexane	1.880	0.938
Water	1.880	0.938
Amphotericin B (positive control)	0.125	0.063

*Bold values indicate the lowest MIC value obtained against each fungus, with the exception of the positive control.

The butanol fraction of *H. natalensis* showed the best antifungal activity against both *A. flavus* and *A. parasiticus* with an MIC value of 0.469 mg/mL (Table 4.1). However, the ethyl acetate fraction showed better antifungal activity against *A. parasiticus* (0.234 mg/mL) when compared to the butanol fraction. The hexane and water fractions only

showed good (< 8 mg/mL) antifungal activity against *A. flavu*s when compared to the other partitioned extracts, which showed noteworthy (≤ 1 mg/mL) activity against both *A. flavus* and *A. parasiticus* (Rios and Recio, 2005; Van Vuuren, 2008).

Based on the results obtained for the antifungal activity of the liquid-liquid partitioned extracts on both *A. flavus* and *A. parasiticus*, the butanol fraction was used for compound isolation using column chromatography. Seven of the major pooled fractions isolated from the butanol fraction of *H. natalensis* showed good antifungal activity against both *A. flavus* and *A. parasiticus* (Table 4.2, Figure 4.5).

Table 4.2: The antifungal activity of the butanol liquid-liquid partitioned pooled fractions from the *H. natalensis* extract, obtained using column chromatography against *A. flavus* and *A. parasiticus*.

	A. flavus		***A. parasiticus***	
Pooled fractions	MIC (mg/mL)	MFC (mg/mL)	MIC (mg/mL)	MFC (mg/mL)
77 - 115	3.75	3.750	1.875	1.875
116 - 128	3.75	3.750	0.234	0.469
129 - 151	1.875	1.875	0.234	0.469
152 - 200	1.875	1.875	**0.117**	0.469
201 - 250	**0.938**	**0.938**	**0.117**	**0.117**
251 - 271	1.875	**0.938**	**0.117**	**0.117**
272 – 286	**0.938**	**0.938**	**0.117**	**0.117**
Amphotericin B (positive control)	0.125	0.125	0.063	0.063

*Bold values indicate the lowest MIC values obtained against each fungus, with the exception of the positive control.

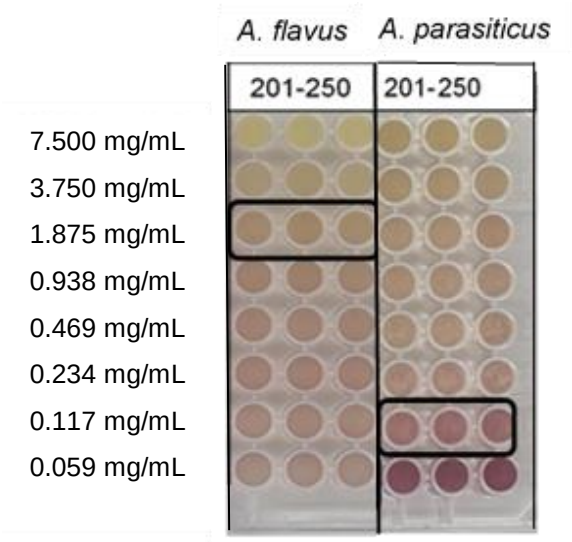


Figure 4.5: Antifungal screening of the butanol fraction of *H. natalensis'* pooled fraction 201-250 against *A. flavus* and *A. parasiticus* using the broth microdilution method.

Noteworthy antifungal activity (≤ 1 mg/mL) against *A. flavus* was observed with the major pooled fractions 201-250 and 272-286. In contrast, pooled fractions 116-128, 129-151, 152-200, 201-250, 251-271 and 272-286 showed noteworthy antifungal activity against *A. parasiticus*. The pooled fractions, which exhibited noteworthy antifungal activity against both *A. flavus* and *A. parasiticus*, were from pooled fractions 201-250 (Figure 4.5) and 272-286, with MIC values of 0.938 and 0.117 mg/mL, respectively. Despite the pooled fractions 201-250 and 272-286 exhibiting noteworthy antifungal activity against both fungal species, their activity against *A. parasiticus* was 8-fold more than the activity observed against *A. flavus*.

It was interesting to note that the antifungal activity of pooled fractions 201-250 and 272-286 (Table 4.2) had better antifungal activity against *A. parasiticus* when compared to the butanol fraction (Table 4.1 and 4.2). In contrast, the butanol fraction had better antifungal activity against *A. flavus* when compared to fraction 201-250 and 272-286 (Table 4.1 and 4.2).

Using GC-MS, compounds from fractions 201-250 were identified. Gas chromatography-Mass spectrometry revealed 111 compounds of which 11 had an area percentage greater than 0.5 % (Appendix A, Figure 8.3, Table 8.2).

Furthermore, there were five major compounds identified whose area percentage was greater than 1 %. The compound which was in abundance in fractions 201-250, was ethanedioic acid (Table 4.3, Figure 4.5), which is also commonly referred to as dibutyl ester. Interestingly, the analysis of the pooled fraction 201-250 detected the presence ethyl acetate and *n*-hexane, which are common solvents generally used as eluents for the isolation of compounds from plants.

Although several studies have reported on the isolation of compounds from *H. natalensis* (leaves, twigs and roots), previously isolated and identified compounds have not been evaluated for their antifungal activity against *A. flavus* and *A. parasiticus* (Van Wyk et al., 1997; Van Vuuren et al., 2007; Mohammed et al., 2009; Henley-Smith et al., 2018). Furthermore, the compounds identified in this study (Table 4.3) have not been previously isolated or detected from *H. natalensis* leaf extracts.

Table 4.3: Compounds identified using Gas chromatography-Mass spectrometry from the pooled fractions 201-250 obtained from the liquid-liquid partitioned extract of *H. natalensis*.

Peak #	Compound name (IUPAC)	Weight (g/mol)	Chemical formula	Area %
11	Ethanedioic acid (dibutyl ester)	202	$C_{10}H_{18}O_4$	23.77
14	Ethyl dimethyl borane	70	$C_4H_{11}B$	9.00
16	tetrahydro-6,6-dimethyl-2H-Pyran-2-one	128	$C_7H1_2O_2$	7.16
92	Diisooctyl phthalate	390	$C_{24}H_{38}O_4$	1.55
47	Hexadecane	226	$C_{16}H_{34}$	1.47

In this study, differences compounds were present in the active fraction 201-250 when compared to those recorded in literature, such as 5-hydroxy-7-methoxy-6-flavanone, aurenticin A, cardamomin, quercetin and 3, 5, 7-trihydroxyflavan (Henley-Smith et al., 2018). This could be due to a number of factors such as the purity of the fractions, the

sensitivity of the compound identification analytical method (in this case GC-MS) and that in this study the ethanolic extract was liquid partitioned before it was subjected to fractionation using column chromatography.

The following section will focus on the antifungal activity of the top five GC-MS identified compounds from the pooled fraction 201-250, with emphasis on species from the *Aspergillus* genera.

Volatile groups of compounds such as esters [dibutyl ester (Figure 4.5)], fatty acids, alcohols and hydrocarbons are some of the compounds, which have been isolated from *A. niger* (Siddiquee et al., 2015). Other ester compounds (isolated from plants such as *Spondias mombin*) including phthalic acid and dodecyl-2-ethyl hexyl ester have been identified as *A. flavus* growth inhibitors (MIC = 1.25 µg/mL) (Osuntokun and Cristina, 2019).

Furthermore, *A. flavus* has been reported to produce dibutyl ester compounds (such as oxalate), especially in Sabouraud liquid media supplemented with acetate (Wilson, 1966). Interestingly, dibutyl ester (ethanedioic acid) was identified as one of the compounds present in abundance (23.77 %) in the *H. natalensis* fraction (fraction 201-250) which had noteworthy (MIC ≤ 1 mg/mL) antifungal activity against *A. flavus* and *A. parasiticus* growth *in vitro* (Wilson, 1966). In addition, dibutyl esters (oxalate compounds) have also been reported as some of the causal agents of some mycotoxin outbreaks, produced by *A. flavus* on substrates such as wheat (*Triticum aestivum*) and oats (*Avena sativa*) (Wilson, 1966). Although, these dibutyl esters have not been evaluated for antifungal activity, they have however been found in plants such as *Nicotiana tabacum* and *Lycopersicum esculentum* (Franceschi and Horner, 1980).

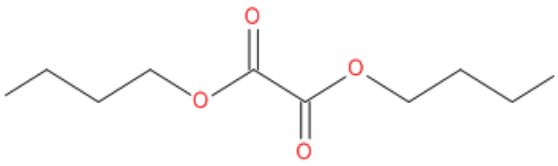


Figure 4.5: The chemical structure of ethanedioic acid

Boronate ester derivatives such as ethyl dimethyl borane (in this study) (Figure 4.6) have been found to have antifungal activity against *A. niger* and *A. flavus* (Irving et al., 2003). Their enhanced biological activity has been attributed to the presence of the pinacol group, whose activity is comparable to boronic acid (food and feed preservative agent) (Irving et al., 2003). Similarly, formyl phenylboronic acid derivatives such as benzylamines have been reported to have fungicidal properties against *A. flavus* and *A. niger* (Klimova et al., 1999). Other borane derivatives such as amine borane have also been reported to inhibit the fungal growth of *A. fumigatus* (Srebnik et al., 2010).

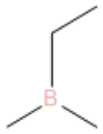


Figure 4.6: The chemical structure of ethyl dimethyl borane

Another compound isolated from pooled fraction 201-250, tetrahydro-6, 6-dimethyl-2H-pyran-2-one (Figure 4.7) is a lactone compound produced by some *Trichoderma* species, which has been associated with the improvement and inhibition of plant growth (Vinale et al., 2008). Although not tetrahydro-6, 6-dimethyl-2H-pyran-2-one (identified in this study), one of its derivatives 6-pentyl-2H-α-one (6PP) is a known antifungal compound, which was reported to have inhibitory activity against aflatoxin-producing *A. flavus* (Cutler et al., 1986).

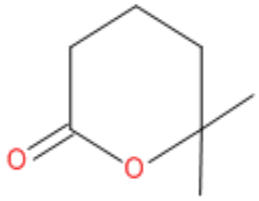


Figure 4.7: The chemical structure of tetrahydro-6, 6-dimethyl-2H-pyran-2-one

Diisooctyl phthalate (Figure 4.8) is a diester and ester phthalate compound. In this study, diisooctyl phthalate was one of the compounds isolated from the butanol fraction of *H. natalensis*. This observation is similar to that noted by Waheed et al. (2019), who isolated the same compound from the butanol fraction of *Ehretia serrata*. Furthermore, Srinivasan et al. (2009) reported that diisooctyl phthalate, isolated from the flowers of *Leea indica*, have antifungal activity against *A. niger*.

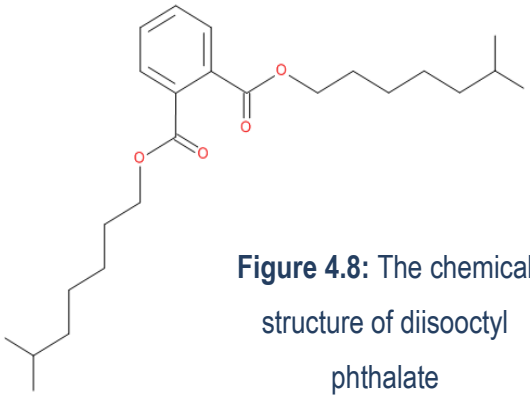


Figure 4.8: The chemical structure of diisooctyl phthalate

Alkane compounds such as hexadecane (Figure 4.9) and heptadecane, isolated from a Nigerian plant-based concoction (Epa-Ijebu) containing plants from *Citrus* spp. and *Afrimonium melagueta*, have been reported to have fungicidal activity against *A. fumigatus* with an MIC value of 50 mg/mL and MFC value of 100 mg/mL (Adeleye et al., 2009). In another study, hexadecane isolated from *Streptomyces cheonanensis* was reported to have antifungal activity against *A. niger* and *A. parasiticus*, with reported MIC values of 64 and 16 µg/mL, respectively, when compared to amphotericin B (16 and 8 µg/mL, respectively) (Muvva et al., 2016).

Figure 4.9: The chemical structure of hexadecane

4.4 Conclusion

The phytochemical analysis of *H. natalensis* extracts conducted by Van Wyk et al. (1997) reported the presence of monoterpenoid compounds. Furthermore, compounds such as lupenone, lupeol and 3*β*-hydroxylup-20(29)-en-28-al and sitost-4-en-3-one were isolated from the hexane root and twig extracts of *H. natalensis* (Mohammed et al., 2009). In addition, the chemical analysis of the leaf extracts (*H. natalensis*), conducted by Shode et al. (2005) and Henley-Smith et al. (2018) revealed the presence of (2E)-2-[(2E)-1-hydroxy-3-phenylprop-2-en-1-ylidene]-5-methoxy-6,6-dimethylcyclohex-4-ene-1,3-dione (also commonly known as ceroptin), 5-hydroxy-7-methoxy-6-flavanone, aurenticin A, cardamomin, quercetin and 3, 5, 7-trihydroxyflavan compounds.

In this study, the above-mentioned compounds were not detected in the pooled fraction 201-250 of the liquid-liquid partitioned butanol extract of *H. natalensis*. The contradicting findings obtained in this study when compared to literature may be attributed to variations in the season and geographic area of plant collection, the age of the tree, the extraction method used, the solvent used as an extractant, how the extract was stored and possibly the analytical method used for the phytochemical analysis of the compounds present in the fraction.

The isolation of the liquid-liquid partitioned extract of *H. natalensis*, through bioassay-guided fractionation indicated two pooled fractions, fractions 201-250 and 272-286, that showed noteworthy (MIC ≤ 1 mg/mL) antifungal activity against *A. flavus* and *A. parasiticus* growth *in vitro*. Furthermore, the phytochemical analysis of the pooled fraction 201-250, indicated the presence of ethanedioic acid, diisooctyl phthalate and tetrahydro-6, 6-dimethyl-2H-pyran-2-one amongst other GC-MS identified compounds. Moreover, the five major compounds identified (based on an area percentage greater than 1 %) and their derivatives have been reported to have antifungal activity against *Aspergillus* species. Therefore, the activity of the *H. natalensis* extract could be due to the combined effect of these to inhibit fungal growth.

Although GC-MS was able to detect compound classes such as phenolics and flavonoids, there may be other compounds present in the pooled fraction 201-250, which GC-MS was unable to detect. Therefore, other analytical methods such as Liquid chromatography tandem-Mass spectrometry (LC-MS/MS) and Nuclear Mass spectrometry (NMR) may be considered for the further identification of compounds.

Chapter 5
The efficacy of extracts from South African indigenous plants as seed treatments against *Aspergillus flavus* on maize

5.1 Introduction

In southern Africa, *Zea mays* (L.) (maize) is a commonly consumed staple food that is used in its original state (fresh) or processed either into cooked, fermented or milled products (Mutamba et al., 2009; Shephard et al., 2012; Hove et al., 2016). In South African rural subsistence farming communities, the ingestion of maize can reach levels of up to 2 kg per person daily (Burger et al., 2010; Alberts et al., 2016). The contamination of maize with fungi and mycotoxins often occurs during preharvest, harvest, post-harvest, transport and in storage (Kurtzman et al., 1987; Klich, 2007a; Shephard et al., 2008; Mutamba et al., 2009; Amaike and Keller, 2011). In Africa, the most prevalent mycotoxins found on maize grain include aflatoxin B_1 commonly produced by *Aspergillus flavus* and *A. parasiticus* and fumonisin B_1 commonly produced by *Fusarium verticillioides* and *F. proliferatum* (Shephard et al., 2007; Alberts et al., 2016; Hove et al., 2016; Mngqawa et al., 2016; Mwalwayo and Thole, 2016; Murashiki et al., 2017).

In developing countries in Asia and Africa, aflatoxin-related outbreaks are a major concern (Yu et al., 2004, Lewis et al., 2005). This is due to various factors such as poor post-harvest practices, poor or lack of proper agronomic practices and a lack of technical resources (Eaton and Groopman, 1994; Sowley, 2016). Other factors include financial and educational resources (e.g. seminars on crop management to the correct target audiences), to ultimately minimize fungal and mycotoxin contamination of grains in storage and on crops in the field (Eaton and Groopman, 1994; Sowley, 2016). The growth of *Aspergillus* species and the production of aflatoxins are often due to direct

contamination of the grains and their survival in storage and during food processing (Park and Stoloff, 1989). In the field, the growth of *Aspergillus* species and production of aflatoxins is affected by many factors, two of the biggest being drought stress and the temperature of the surrounding crops in the field (Klich, 2007a).

Large amounts of grains produced are often unsuitable for human consumption due to contamination by *Aspergillus* species and the subsequent production of aflatoxins (Janardhana et al., 1999; Probst et al., 2010). The occurrence of aflatoxins (43.75 %) was reported to have been mainly found in maize samples from South Africa, Kenya, Lesotho, Egypt, Ethiopia and Togo, amongst other African countries (Darwish et al., 2014). Some of the major factors contributing to the contamination and predisposition of grains to *A. flavus* growth and aflatoxins are usually beyond the farmers' control. Abiotic factors, such as the susceptibility of the cultivar, mechanical damage to the grains (caused by insects), excessive rainfall, moisture and other crop stresses have an effect, which may also lead to the production of mycotoxins in or on these grains (CAST, 1989; Robens, 1990; Williams et al., 2004).

Control methods of *Aspergillus* contamination, on crops in the field and stored grains, include the use of physical methods, synthetic chemical fungicides and biological controls (Payne, 1998; El-Kady et al., 2014). Synthetic chemical fungicides include the use of kojic acid, synthetic fungicides high in acetic acid, formaldehyde and ammonium (El-Kady et al., 2014). Biological control often includes the use of avirulent and atoxigenic strains of *A. flavus* (Payne, 1998).

In addition, Suttajit (1989) described the reduction of grain contamination with aflatoxins as a three-step process, consisting of primary, secondary and tertiary steps of reduction. The primary reduction methods were described to include treating grains with fungicides, planting cultivars resistant to either fungal contamination or aflatoxin production and storing grains at low moisture contents and low temperatures (Suttajit, 1989; Sowley, 2016). Removing, detoxifying and inactivating contaminated seeds makes up some of the secondary reduction methods while the tertiary reduction methods were described to be destroying the contaminated grains until no mycotoxins could be detected (Suttajit, 1989; Sowley, 2016).

Unfortunately, excessive use of fungicides on crops and as stored seed treatments has contributed to the resistance of fungal pathogens to antifungals (Williams, 2006; Da Cruz Cabral et al., 2013). As a consequence of this, higher concentrations of antifungal agents are being used, which in turn increases the toxic residual effects associated with their use on the environment, food and human health (Williams, 2006; Etcheverry et al., 2009; Da Cruz Cabral et al., 2013).

Various studies have used plant extracts and essential oils as seed treatments against the contamination of stored grains with *A. flavus* (Reddy et al., 2009; El-Habib, 2012; Chaudhari et al., 2018; Jimenez et al., 2018). For example, Reddy et al. (2009) reported that aqueous extracts of *Syzygium aromaticum* (clove), used as rice (*Oryza sativa*) seed treatments, were able to completely inhibit the growth of *A. flavus*. Similarly, wheat (*Triticum aestivum*) and chickpea (*Cicer arietinum*) seeds treated with cumin (*Cuminum cyminum*) essential oils and then stored for one year were able to provide 65.8 and 75.0 % protection against *A. flavus*, respectively (Kedia et al., 2014). Jimenez et al. (2018) reported that the diethyl ether fraction of the ethanolic leaf extract of *Zuccagnia punctata,* as a seed treatment against *A. flavus* growth on maize grains had seed protective properties comparable to a known seed-borne fungicide combination made up of thiram and carbendazim.

Therefore, in an attempt to reduce the increasing public concerns associated with the use of synthetic chemicals, alternative treatments such as the use of plants as natural antifungals, is increasingly being studied (Da Cruz Cabral et al., 2013; Boukaew et al., 2017). Plants generally produce a wide variety of compounds for their development or as a result of stressors or attacks by pathogens (Van Vuuren et al., 2007; Da Cruz Cabral et al., 2013; Ruchika et al., 2019). The relative safety, environmentally friendly, reduced phytotoxicity, easy biodegradability of plant extracts, and phytochemicals contributes to some of the advantages of using plant extracts as antifungals (Da Cruz Cabral et al., 2013; Tian et al., 2018).

Previously, the leaf extracts of *Erythrophleum lasianthum*, *Heteropyxis natalensis* and *Warburgia salutaris* extracted using ethanol, water and acetone, respectively, showed noteworthy (MIC≤ 1 mg/mL) antifungal activity against *A. flavus in vitro* (Chapter 3

section 3.3.1). Therefore, the aim of this chapter was to evaluate the potential of the three plant extracts as seed treatments against *A. flavus* growth on maize seeds stored for a period of three and six months.

5.2 Materials and Methods

5.2.1 Materials

Untreated maize seed was obtained from Pannar, KwaZulu-Natal, South Africa. The seed was stored in a plastic container at ± 5 °C until use. All reagents used, unless stated otherwise, were purchased from Sigma-Aldrich/Merck (Johannesburg, South Africa).

5.2.2 Plant collection and extraction

The leaves were collected from the Manie van Schijff Botanical Garden at the University of Pretoria, Pretoria, South Africa (January 2018). Herbarium specimens of the three plants were submitted to the H.G.W.J. Schweickerdt Herbarium at the Department of Plant and Soil Sciences, University of Pretoria, for identification and voucher numbers were assigned (Chapter 3 section 3.2.1). The plant extracts of *E. lasianthum* (ethanol), *H. natalensis* (water) and *W. salutaris* (acetone) were prepared as discussed in Chapter 3 section 3.2.2 and stored in polytops at ± 5 °C until use.

5.2.3 Pre-seed treatment optimization

Prior to the seed treatment experiments, various preliminary trials were conducted to determine the optimum concentrations of the plant extracts needed for seed treatment and the best method of extract application. A more suitable method of artificially inoculating the maize seeds was investigated by comparing different methods of artificial inoculation. These methods included the artificial inoculation of all trial seeds with 0.5 Fungi McFarland (~1.5 $\times 10^8$ cfu/mL) *A. flavus* and using artificially inoculated 0.5 Fungi McFarland spiked seeds (15 seeds per treatment). Following the various preliminary trials, the optimum plant extract concentrations (50 and 100 mg/mL), method of extract application and the most suitable method of artificially inoculation.

5.2.4 Seed treatment and shelf-life

The three extracts that showed the best antifungal activity *in vitro* were evaluated for their potential as seed treatments. These were *E. lasianthum* ethanol, *H. natalensis* water and *W. salutaris* acetone. The maize seeds (7500) were surface sterilized by immersion in 1 % sodium hypochlorite (NaHClO) solution for 10 minutes, rinsed three times with sterile distilled water (SdH_2O) and then dried for 16 hours on sterile paper towels in a laminar flow cabinet. The seeds were grouped into the different seed treatments, initially into two seed batches: one batch to be spiked with artificially inoculated seeds (0.5 Fungi McFarland spore suspension ~1.5 $\times 10^8$ cfu/mL) and the other batch of seeds were uninoculated.

The one seed batch was spiked with 15 artificially inoculated seeds (per treatment) for 10 minutes. After spiking the seeds, the artificially inoculated seeds were discarded. The spiked and the control seed batches were further grouped into the different seed treatments and treated with 50 and 100 mg/mL of the plant extracts dissolved in 10 % dimethyl sulphoxide (DMSO), for 30 minutes. The positive control used was Captan (0.01 mg/mL) (a synthetic fungicide), while the negative control used was 10 % DMSO. Following treatment, the seeds were left to dry for 16 hours on sterile paper towels in a laminar flow cabinet (± 25 °C). One hundred seeds from each treatment were directly plated onto Petri dishes containing PDA (five seeds per dish) while the other seeds (seeds for storage) were packaged in sealed plastic bags (60 micron) (Figure 5.1) and stored in the dark for three and six months at 25 ±1 °C. After each storage period, the treated seeds were plated onto PDA (Figure 5.2) and the fungal incidence was evaluated after a 5-day incubation period at 25 ±1 °C. The procedure was repeated in two independent experiments. The incidence of *A. flavus* on the treated seeds in comparison to the untreated seeds was converted to percentage fungal growth inhibition (Mohana and Raveesha, 2007) using the following equation:

$$\%\ \text{fungal inhibition} = \frac{\mathrm{dc} - \mathrm{dt}}{\mathrm{dc}} \times 100$$

Where dc = average fungal incidence in the untreated control, dt = average fungal incidence in the treatment

Figure 5.1: Plant extract treated maize seed packaged in sealed plastic bags for storage.

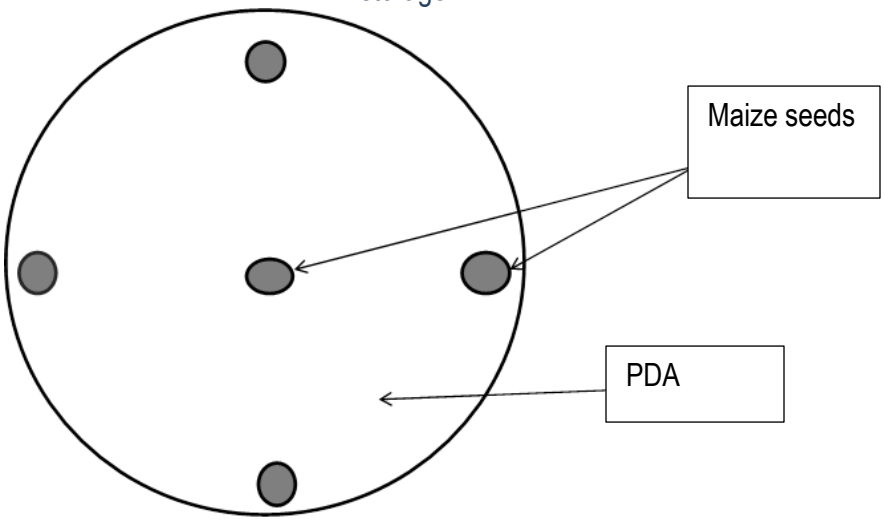


Figure 5.2: An illustration of the seed arrangement of the plant-extract treated seeds after each storage period, for the evaluation of fungal incidence.

5.2.5 Statistical analysis

The average fungal incidence values per replicate were further analyzed using Graphpad Prism 4, statistical analysis software. This was done to determine the significant differences between the percentage growth inhibition for each seed treatment (One-way ANOVA) and the significant differences of the efficacy of each extract over a period of six months (Two-way ANOVA). A *p*-value <0.05 was considered to be

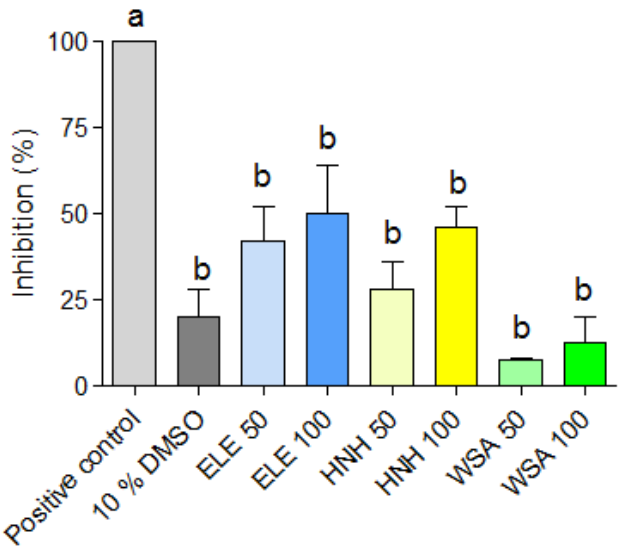
statistically significant. A Tukey's multiple comparison test was used as a post-hoc analysis, comparing the different columns with one another.

5.3 Results and Discussion

There is a lack of relevant literature on plant extracts as seed treatments on maize seeds against *A. flavus* and moreover, the use of the selected plant extracts as seed treatments.

The percentage inhibition of *A. flavus* on maize seeds treated with 50 and 100 mg/mL extract concentrations of *E. lasianthum*, *H. natalensis* and *W. salutaris* showed a reduction in the incidence of *A. flavus* over a period of six months. In addition, the extracts showed no observable negative effects on seed germination.

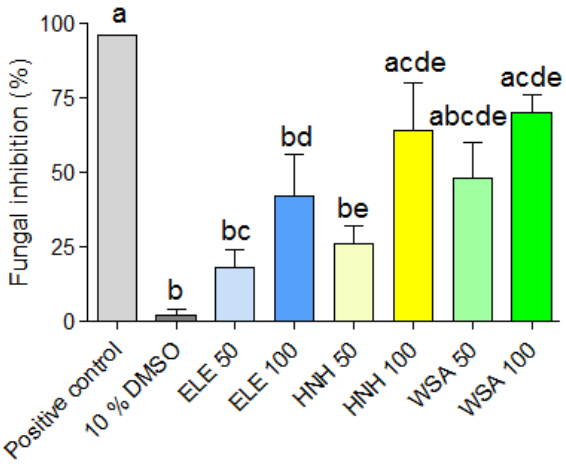
The inhibition of *A. flavus* on maize seeds treated and plated out prior to storage was not significantly reduced by any of the extracts at 50 and 100 mg/mL extract concentrations, when compared to the positive control (Captan) (Figure 5.3). Although Captan is not commonly used as a maize seed treatment, it has been used as a soybean (*Glycine max*) seed treatment against *A. flavus* growth, in storage (Krishnamurthy and Shashikala, 2006; Mahal, 2014). Furthermore, among the different plant extracts used, no significant differences in fungal growth inhibition were observed at either 50 or 100 mg/mL.



*Positive control: Captan, ELE: *E. lasianthum* ethanol, HNH: *H. natalensis* water, WSA: *W. salutaris* acetone, 50: 50 mg/mL, 100: 100 mg/mL.

Figure 5.3: The effect of three plant extracts as seed treatments against *A. flavus* incidence, indicated as percentage fungal inhibition after 16 hours of treatment. The data is represented as the mean ± SEM, One-way ANOVA, $P \leq 0.05$, n=4. Tukey's multiple comparison test. Values of bars followed with a different letter are significantly different.

After the three month storage period (Figure 5.4), the incidence (%) of *A. flavus* on the treated maize seeds was reduced when compared to the treated seeds analyzed prior to storage (Figure 5.3). Furthermore, amongst the plant extract treated seeds, the highest fungal inhibition was observed with the *W. salutaris* extract (100 mg/mL) which was able to inhibit 70.5 % fungal growth while the least fungal growth inhibition was observed with the *E. lasianthum* extract at 50 mg/mL, which only inhibited 19.5 % fungal growth (Figure 5.4). The fungal growth inhibition on seeds treated with 100 mg/mL of *H. natalensis* (65.0 %) and *W. salutaris* (70.5 %) were significantly different from the seeds treated with 10 % DMSO.



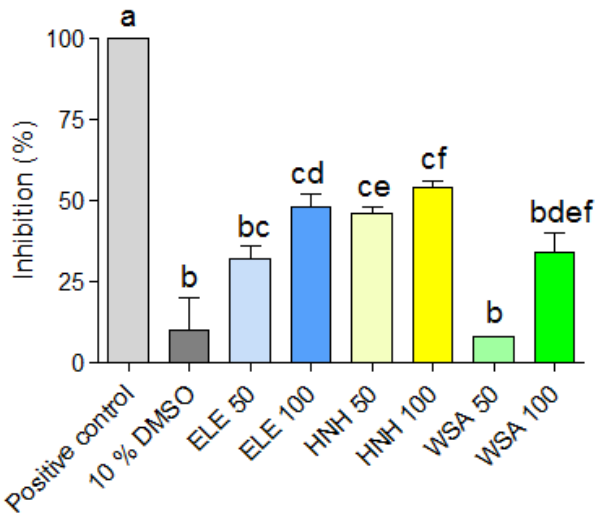
*Positive control: Captan, ELE: *E. lasianthum* ethanol, HNH: *H. natalensis* water, WSA: *W. salutaris* acetone, 50: 50 mg/mL, 100: 100 mg/mL.

Figure 5.4: The effect of three plant extracts as seed treatments against *A. flavus* incidence on maize seeds after three months of storage, indicated as percentage fungal inhibition. The data is represented as the mean ± SEM, One-way ANOVA, $P \leq 0.05$, n=4. Tukey's multiple comparison test. Values of bars followed with a different letter are significantly different.

Seeds treated with *H. natalensis* (100 mg/mL) and *W. salutaris* (50 and 100 mg/mL) were not significantly different from the positive control (Captan) (Figure 5.4). *Warburgia salutaris* (50 mg/mL) also showed no significant difference to the negative control (Figure 5.4). Similarly, Iram et al. (2018) noted that aqueous extracts of *Eucalyptus citriodora*, *Trachyspermum ammi* and *Ocimum basilicum* as seed treatments (at 10 % extract concentrations v/v) on stored maize seeds, were able to inhibit *A. flavus* growth by 100, 91 and 83 %, respectively. It was interesting to note that the water extract (*H. natalensis*) at 100 mg/mL (Figure 5.4), had better, although not significant fungal growth inhibition when compared to the organic solvents extracts (*E. lasianthum* extract). Water as an extractant has been reported to extract fewer compounds (such as tannins, anthocyanins, saponins and terpenoids) present in plants, when compared to organic solvent-extracted extracts (such as methanol and ethanol, which extract alkaloids, flavones and polyphenol compounds in addition to those extracted with water) (Cowan, 1999). Some fungicides such as Prochloraz, which are often used as *Aspergillus* spp. growth inhibitors, contain polyphenol compounds (Wink et al., 2012;

Ansari et al., 2013; Yang and Zhang, 2019). These polyphenol compounds aid in the inhibition of transporter proteins (ABC), which are responsible for fungal pathogen resistance to fungicides and seed treatments (Wink et al., 2012; Ansari et al., 2013; Yang and Zhang, 2019).

The growth inhibition of *A. flavus* on plant extract treated maize seeds after the six month storage period, was significantly inhibited by *E. lasianthum* at 100 mg/mL and *H. natalensis* at 50 and 100 mg/mL extract concentrations when compared to the negative control (Figure 5.5). The fungal growth inhibition percentages of *E. lasianthum* (100 mg/mL) and *H. natalensis* (50 and 100 mg/ml) were 48, 47 and 55 %, respectively. In contrast, *W. salutaris* (50 mg/ml), was unable to inhibit fungal growth when compared to the 10 % DMSO treated seeds. Although, *W. salutaris* (50 mg/mL) did not show any significant differences to 10 % DMSO, *W. salutaris* at 100 mg/mL was able to inhibit fungal incidence comparable to both *E. lasianthum* (100 mg/mL) and *H. natalensis* (50 and 100 mg/mL).



*Positive control: Captan, ELE: *E. lasianthum* ethanol, HNH: *H. natalensis* water, WSA: *W. salutaris* acetone, 50: 50 mg/mL, and 100: 100 mg/mL.

Figure 5.5: The effect of three plant extracts as seed treatments against *A. flavus* incidence on maize seeds after six months of storage, indicated as percentage fungal inhibition. The data is represented as the mean ± SEM, One-way ANOVA, $P \leq 0.05$, n=4. Tukey's multiple comparison test. Values of bars followed with a different letter are significantly different.

The differences in growth inhibition of *A. flavus* during the different storage periods may be attributed to various factors. These factors could have been the concentration of the extract, the moisture content of the maize seeds, the storage conditions and the storage period (Prakash et al., 2012; Tian et al., 2012). The antifungal potential of the plant extracts has also been reported to be dependent on the chemical composition of the plants themselves. For example, Sharma and Sharma (2012) reported that *Lawsonia inermis* and *Murraya paniculata* leaf extracts were effective seed protectants against *A. flavus* and *A. parasiticus* on freshly harvested and six month stored maize seeds treated with extract concentrations of 33-40 mg/g and 40-45 mg/g, respectively. The efficacy of *L. inermis* and *M. paniculata* leaf extracts was higher at lower extract concentrations (40 mg/g) against *A. flavus* on maize seeds when compared to the extract concentrations of 50 and 100 mg/mL in this study. This also indicates, in general, that there are factors other than controlled storage environments and low moisture contents, which contribute to effective fungal growth inhibition on plant extracts

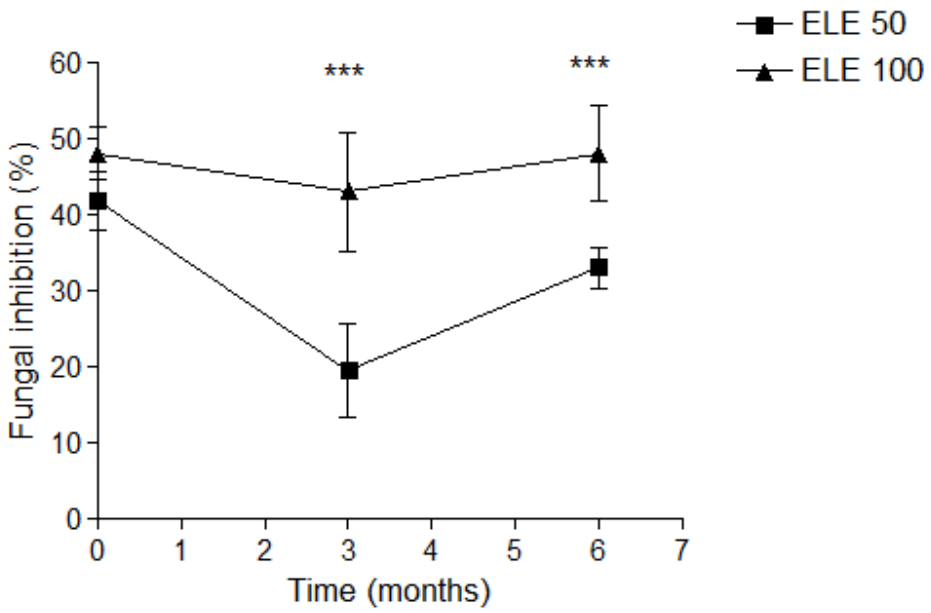
treated and then stored maize seeds. Although, these factors may affect the efficacy of extracts, as seed treatments to a certain extent, it appears that the storage time and concentrations of the extract play an important role regardless of the storage conditions.

The efficacy of the extract may be due to the solvent used for extracting compounds from the plant. Often polar solvents are used for extracting the polyphenols from plants (Dai and Mumper, 2010). The standard solvent, which is considered most suitable, is ethanol, although aqueous mixtures of ethanol, methanol, acetone and ethyl acetate are also considered suitable for plant extraction depending on the target compounds (Dai and Mumper, 2010). This is noteworthy and in the present study, it was observed that after the six month storage period, the water extract of *H. natalensis* had the most significant fungal incidence reduction when compared to the organic solvent-extracted extracts of *E. lasianthum* and *W. salutaris*. It would have been expected that the acetone or ethanolic extract would have better fungal growth inhibiting properties, when compared to the water extract. This is because polyphenols such as caffeic acid, ferulic acid, benzoic acid, gallic acid and vanillic acids are some of the polyphenolic compounds, which have been previously reported as *Aspergillus* species growth inhibitors on food and feed, and which ethanol as an extractant is able to extract from plants (Bilgrami et al., 1980; Chipley and Uraih, 1980; Zaika and Buchanan, 1987; Gouraman and Bullerman, 1995; Aziz et al., 1998; Jackson and Bullerman, 1999; Shi et al., 2005).

The efficacy of the extracts used as seed treatments over a period of six months was evaluated to determine the time at which the optimum activity against *A. flavus* incidence occurred (Figure 5.6, 5.7 and 5.8).

The *E. lasianthum* extracts, regardless of concentration, effectively reduced the incidence of *A. flavus* on maize seeds after a period of six months (Figure 5.6). However, the storage period affected its activity. Figure 5.6 indicates that the longer *E. lasianthum* extract treated seed was stored, the better its efficacy in reducing fungal incidence. This however contradicts Marks and Stroshine's (1995), statement that longer storage periods, often result in lower subsequent storability of the product and therefore decreased efficacy or biological activity. However, at the three month storage

period, the reduction in fungal incidence was not as effective when compared to the six month storage period. Marks and Stroshine (1995) further postulated that the decrease in biological activity on stored products might be due to factors such as the moisture content. Using Two-way ANOVA and the Bonferroni post-hoc test, the extract concentration was determined to be the main factor influencing variance in the efficacy of *E. lasianthum* in reducing fungal incidence. Extract concentration contributed 94.30 % to the variance observed, while the storage period contributed 2.89 % of the variance and the interaction between the fungus and the storage period accounted for 1.36 % of the variance.

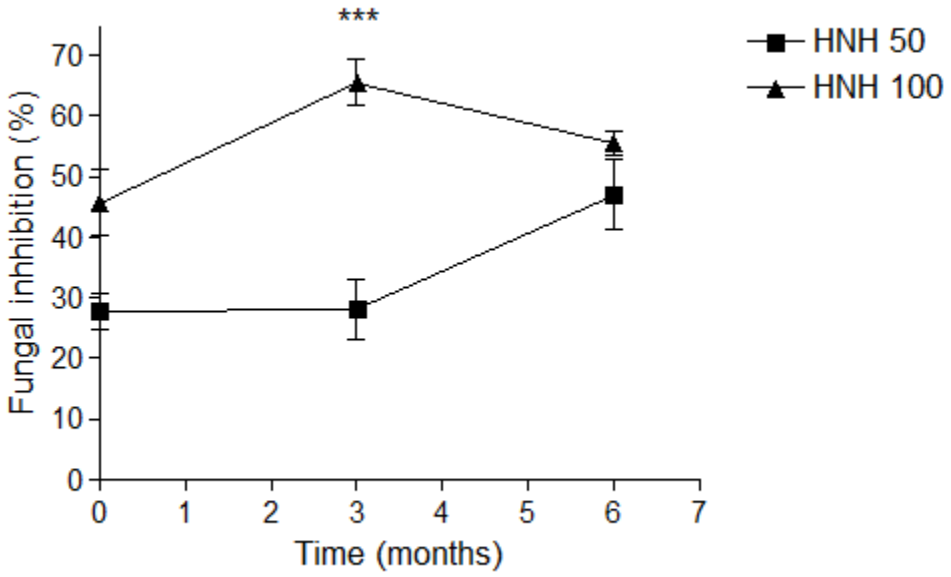


*ELE: *E. lasianthum* ethanol extract, 50: 50 mg/mL, and 100: 100 mg/mL.

Figure 5.6: The effect of the different concentrations of *E. lasianthum* extracts as seed treatments on maize seed against *A. flavus* over a period of six months. The data is represented as the mean ± SD, Bonferroni posttest, Two-way ANOVA, P < 0.0001, n=4. *** above the error bars indicate significant differences.

At 100 mg/mL, the *H. natalensis* extract (Figure 5.7) exhibited, good fungal growth reduction/inhibition after three months in storage. Thereafter the fungal incidence increased slightly after an additional three month storage period.

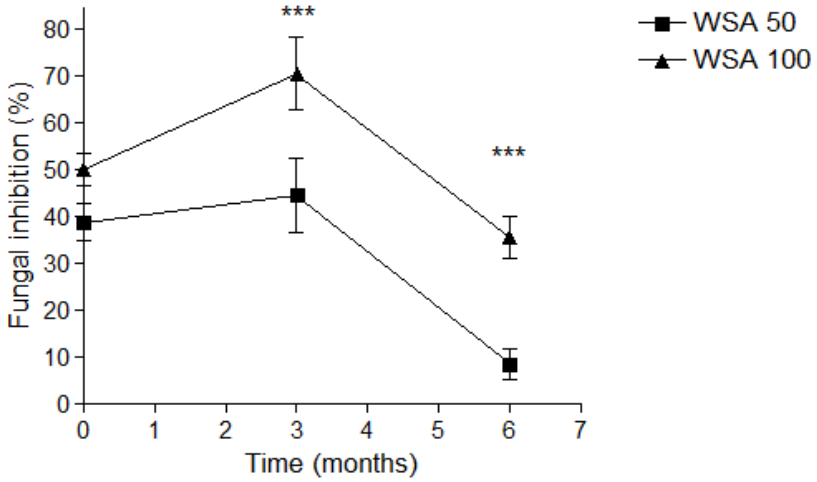
The *H. natalensis* extract at 50 mg/mL was most effective for longer storage periods when compared to the extract at higher concentrations. This therefore may suggest that the extract remained relatively stable despite the decrease in the reduction of fungal incidence observed at extract concentrations of 100 mg/mL at the six month storage period. Other factors to consider, which may have influenced the efficacy could have been the moisture content of the seeds and the humidity within the storage place (Hell et al., 2010; Villers et al., 2014). According to the Bonferroni post-hoc test, the storage period only accounted for 0.42 % of the variance of the extract's ability to reduce fungal incidence, which is considered not as significant when compared to the concentration (94.61 %).



*HNH: *H. natalensis* water extract, 50: 50 mg/mL, and 100: 100 mg/mL.

Figure 5.7: The effect of the different concentrations of *H. natalensis* extracts as seed treatments against *A. flavus* over a period of six months. The data is represented as the mean ± SD, Bonferroni posttest, Two-way ANOVA, P < 0.0001, n=4. *** above the error bars indicate significant differences.

The *W. salutaris* extract as a seed treatment (Figure 5.8), was observed to be most effective against *A. flavus* at both 50 and 100 mg/mL extract concentrations after three months of storage. However, the effect thereafter decreased with an increase in storage period. This observation is similar to Marks and Strochine's (1995) statement that longer storage periods result in decreasing product storability and efficacy or biological activity. Although this trend is observed for both the 50 and 100 mg/mL concentrations, the reduction in fungal incidence is still most effective at the highest concentration of 100 mg/mL (at the three month period). Kapoor et al (2007) and Negi (2012) postulated that the reduced efficacy of crude extracts against some pathogenic fungi, in general, is due to the presence of flavonoids present in the form of glycoside sugars, which mainly contribute to the decrease in the crude extract's efficacy when compared to pure compounds. Temba et al. (2017), in addition, stated that the chemical and physical composition of stored products also greatly influences the storability of these products, which may result in the products remaining stable over longer periods of time or degrading with increasing storage time.



*WSA: *W. salutaris* acetone extract, 50: 50 mg/mL, and 100: 100 mg/mL.

Figure 5.8: The effect of the different concentrations of *W. salutaris* extracts as seed treatments on maize seed against *A. flavus* over a period of six months. The data is represented as the mean ± SD, Bonferroni posttest, Two-way ANOVA, P < 0.0001, n=4. *** above the error bars indicate significant differences.

Griggs et al. (2001) and Laher et al. (2013) hypothesized that chemical constituents present in plants are known to vary due to both adverse and favourable factors, which in turn may also have effects on the biological activity in storage. Griggs et al. (2001) claimed that the shelf-life or stability of plant extracts, especially when dry, does not change in storage based on the plant species. The results obtained for *E. lasianthum* (50 mg/mL), *H. natalensis* (50 mg/mL) and *W. salutaris* (50 and 100 mg/mL), as seed treatments, over the six month period refutes this statement. However, Stafford et al. (2005) reported that the shelf-life of extracts varies depending on the plant part used to make the extract. Plant parts such as the roots, bark and tubers or bulbs often have a longer shelf-life, because these plant parts are stable in assimilating and storing produced secondary metabolites when compared to the leaves (Stafford et al., 2005). Therefore, the efficacy of the three plant extracts as seed treatments is dependent on various factors, with its stability being the main contributor to its efficacy or activity in storage.

5.4 Conclusion

This is the first report of the three selected plant extracts being used as seed treatments against *A. flavus* incidence on maize seeds over a storage period of six months. The selected plants, *E. lasianthum*, *H. natalensis* and *W. salutaris* possess a variety of antifungal compounds (Gundidza et al., 1993; Orsini et al., 1997; Nielsen et al., 2012; Samie and Mashua, 2013; Henley-Smith et al., 2018), which could possibly reduce *A. flavus* incidence on maize seeds in storage without having negative observable effects on seed germination. The plant extract, which exhibited the most fungal growth inhibition, was the water extract of *H. natalensis* at 100 mg/mL. Furthermore, the efficacy of the three extracts as seed treatments over the six month storage period was found to be mainly concentration dependent.

Future research may consider the evaluation of the phytotoxicity of the three plant extracts, use of different plant parts and the evaluation of these plant extracts as cost effective and safer seed treatments than synthetic preservatives. Such research could lead to the decreased use of synthetic chemical fungicides, which are associated with

harmful residual effects. Further studies can also focus on the optimization of the extract concentrations in order to achieve complete fungal growth inhibition.

Chapter 6

General Discussion

6.1 Acheivement of objectives

The antifungal activity of the three plants, *Erythrophleum lasianthum*, *Heteropyxis natalensis* and *Warburgia salutaris* (acetone, ethanol and water extracts) was determined against *Aspergillus flavus* (aflatoxin-producing) and *A. parasiticus* isolates. Although the water extracts of *E. lasianthum* and *W. salutaris* showed poor antifungal activity against *A. flavus* and *A. parasiticus*, at the highest tested concentration, the organic extracts had good antifungal activity (< 8 mg/mL). Overall, the acetone extract of *W. salutaris* showed the best antifungal activity of 0.117 mg/mL, which was less than the activity exhibited by Amphotericin B (positive control) (MIC= 0.250 mg/mL).

Based on the findings of the initial antifungal screening of all the plant extracts, the aflatoxin reduction potential of *E. lasianthum* (ethanol), *H. natalensis* (water) and *W. salutaris* (acetone), was evaluated. These three extracts were able to reduce aflatoxin B_1 (AFB_1); however, *W. salutaris* reduced the most AFB_1 by up to 49 % at 75 mg/mL extract concentration. The water extract of *H. natalensis* showed intermediate AFB_1 reduction when compared to *W. salutaris* and *E. lasianthum*, with 26.38 and 42.82 % at extract concentrations of 15 and 75 mg/mL, respectively.

The cytotoxicity of *E. lasianthum* (ethanol), *H. natalensis* (acetone, ethanol and water) and *W. salutaris* (acetone) was evaluated on the HeLa and HepG2 cell lines. *Heteropyxis natalensis* (water) and *W. salutaris* (acetone) had moderate ($50 \leq IC_{50} \leq 100$ µg/ml) toxicity on the HeLa cells when compared to their toxicity on the HepG2 cells. The ethanolic extracts of *E. lasianthum* and *H. natalensis* showed, overall, the highest safety ($IC_{50} > 100$ µg/ml) on both cell lines.

Based on the national status of plant conservation [*E. lasianthum*: Near threatened, *W. salutaris*: Endangered and *H. natalensis*: Least concern (Foden et al., 2005,

Williams et al., 2008a, Williams et al. 2008b)] and results from the *in vitro* antifungal activity and AFB_1 reduction, *H. natalensis* was selected for compound isolation using column chromatography. The fractions collected from *H. natalensis*, pooled into 25 sub-fractions were evaluated for their antifungal activity against *A. flavus* and *A. parasiticus*. The sub-fractions containing fractions 201-250 and 272-286 exhibited noteworthy activity against both fungal species. Using Gas chromatography-Mass spectrometry (GC-MS) five compounds with an area percentage greater than 1 % were identified as the main contributors responsible for antifungal activity against *A. flavus* and *A. parasiticus*. These compounds have been previously reported to have antifungal activity against some *Aspergillus* species (Wilson, 1966; Osuntokun and Cristina, 2019).

The *E. lasianthum* (ethanol), *H. natalensis* (water) and *W. salutaris* (acetone) extracts as seed treatments on maize seeds *in vitro*, over a period of six months in storage, were able to reduce *A. flavus* incidence without any observable effects on seed germination. Although, the directly treated seeds showed no significant differences from the negative control (10 % DMSO), with increasing storage period, the efficacy of the extracts against *A. flavus* incidence in storage increased. Although complete (100 %) fungal growth inhibition, as observed with the positive control is what one would desire, a reduction in fungal incidence indicates that optimizing the extract concentration may potentially lead to complete fungal growth inhibition. Furthermore, after the six month storage period, the best fungal incidence reduction was exhibited by the *H. natalensis* extract at both 50 and 100 mg/mL. This study represents the first report of these plant extracts being used as seed treatments against *A. flavus* growth in stored maize seed.

The results obtained in this study further indicated that the antifungal activity of the selected plants, with emphasis on the *E. lasianthum* (ethanol), *H. natalensis* (water) and *W. salutaris* (acetone) extracts, may vary *in vivo* when compared to the activity *in vitro*. According to Polak (1998), the antifungal activity (*in vitro*) does not always correspond completely to the activity noted *in vivo*. Furthermore, the activity noted *in vivo* may be less effective, ineffective or excessively greater than the activity observed *in vitro* (Polak, 1998). This may be due to the controlled concentration of the pathogen (spore

suspension) being favourable for *in vitro* studies when compared to the concentration of the pathogen *in vivo*, which is due to natural contamination of seed.

Although there are no previous reports from literature on these plant extracts as antifungal agents, AFB_1 reducers and seed treatments against *A. flavus* and *A. parasiticus in vitro*, the results from this study indicate that these plants are potential biocontrol agents against *A. flavus* and *A. parasiticus*. A plant-based seed treatment with antifungal growth and AFB_1 production reduction against aflatoxin-producing *Aspergillus* species would substantially benefit the smallholder and/or subsistence farmers as well as contribute to the decrease in the harmful residual effects associated with the use of synthetic chemical fungicides.

Moreover, the outcomes of this study provide an understanding on how plants and their chemical constituents have the potential to inhibit *Aspergillus* species growth and the production of AFB_1 *in vitro*. The consumption of fungi and aflatoxin contaminated feed and foodstuff can cause health problems and result in the loss of food security. It is therefore necessary that *H. natalensis*, its isolated compounds and other South African indigenous plants, be evaluated for their antifungal and anti-mycotoxigenic effects against other mycotoxigenic fungi contaminating cereal grains and crops. This study provides a basis for developing strategies in which fungal infestations on maize seed can be reduced, especially during storage. Plant extracts as control agents are more feasible for storage environments when compared to use in the field. In the field, plant extracts may not provide substantial protection against fungal pathogens. This may be due to various factors such as the climate or temperature of the environment, which may lead to the degradation of the plant extract constituents responsible for inhibiting fungal growth. In addition, the use of plant extracts as fungal control agents in storage environments can potentially reduce the dependency on synthetic chemical fungicides as fungal control agents.

6.2 Future research

Although literature about toxicity and the mode of action of AFB_1 on humans and animals is known, the phytotoxicity on cereal grain crops such as maize may be

investigated. Furthermore, the mechanism of action of *H. natalensis* should be elucidated as indicated by the antifungal, aflatoxin B_1 reduction and fungal incidence reduction activity reported in this study. The concentration of these plant extracts as seed treatments needs to be optimised in order to achieve complete fungal incidence (100 %) reduction. The stability, mutagenicity and phytotoxicity of these extracts need to be evaluated further for their formulation into or as plant-based fungicides for the control of *A. flavus* and *A. parasiticus* growth on stored grains or on food products.

Chapter 7
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Chapter 8
Appendix A

Table 8.1: Aflatoxin B_1 reduction wet and dry mycelial mass of *Aspergillus flavus* after a 15-day incubation period in Czapek Dox broth at 28 ± 2 °C. The data is represented as the mean and standard deviation of two trials.

	Wet mycelial weight		Dry mycelial weight	
Treatment	**Mean**	**SD**	**Mean**	**SD**
positive	7.92	0.08	0.15	0.01
	7.63	0.09	0.21	0.01
Negative	8.80	0.65	0.21	0.01
	8.20	0.62	0.24	0.00
ELE 15	6.87	0.14	0.25	0.01
	7.25	0.31	0.28	0.01
ELE 30	7.19	0.14	0.37	0.00
	2.93	0.04	0.32	0.00
ELE 75	2.97	0.07	0.24	0.00
	13.09	0.17	0.35	0.00
HNH 15	12.36	0.12	0.28	0.01
	13.63	0.37	0.16	0.01
HNH 30	12.37	0.89	0.27	0.00
	13.06	0.53	0.18	0.00
HNH 75	14.23	0.11	0.47	0.00
	12.55	0.12	0.31	0.00
WSA 15	11.12	0.10	0.56	0.00
	11.88	0.07	0.28	0.01
WSA 30	13.08	0.06	0.28	0.01
	12.58	0.44	0.21	0.01
WSA 75	13.98	0.11	0.71	0.16
	13.65	0.13	0.24	0.01

*ELE: *E. lasianthum* ethanol, HNH: *H. natalensis* water, WSA: *W. salutaris* acetone, 15: 15 mg/mL, 30: 30 mg/mL and 75: 75 mg/mL.

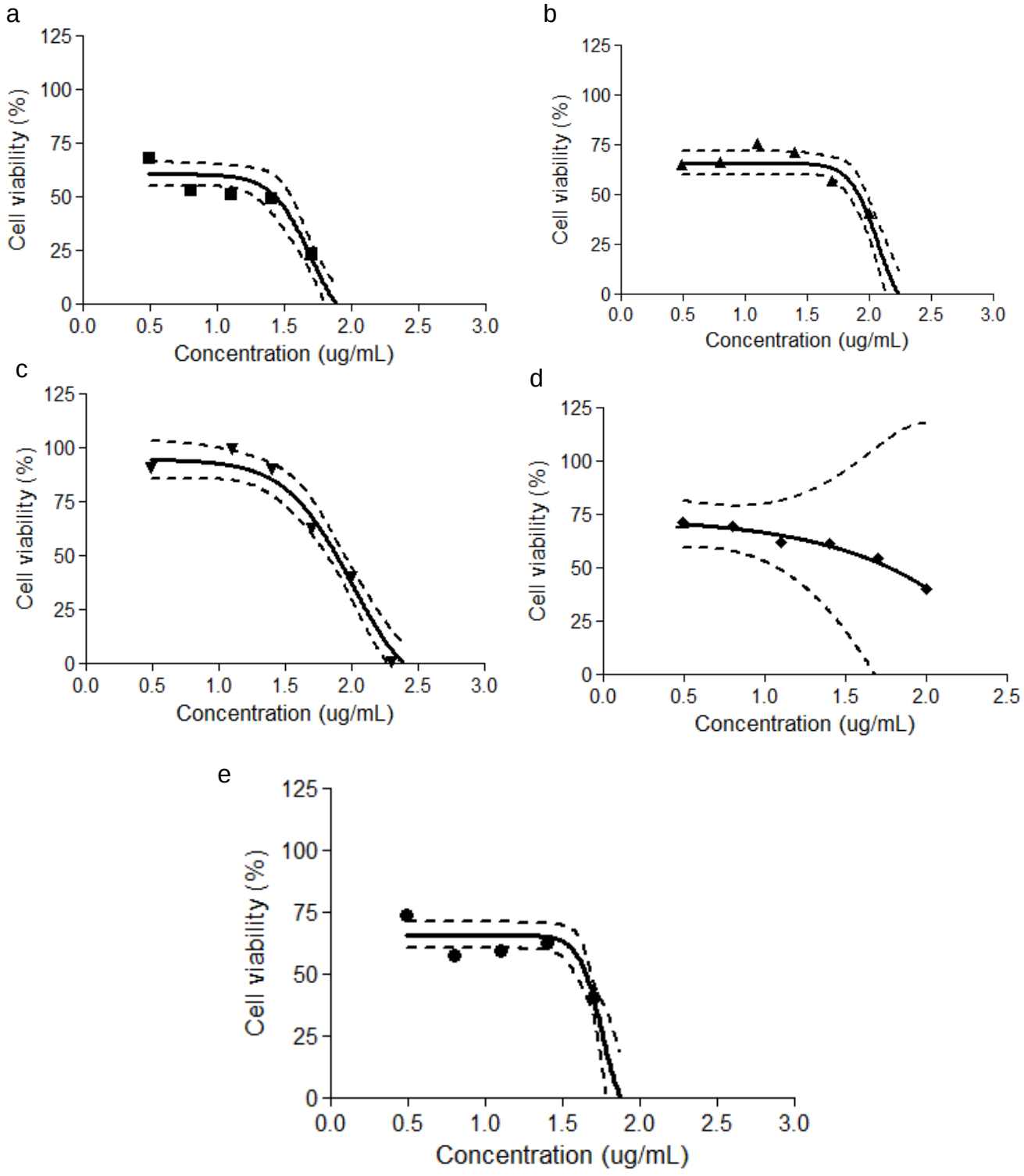

Figure 8.1: Cell viability graphs of selected plant extracts a) *E. lasianthum* ethanol, b) *H. natalensis* acetone, c) *H. natalensis* ethanol, d) *H. natalensis* water and e) *W. salutaris* acetone on the HeLa cell line.

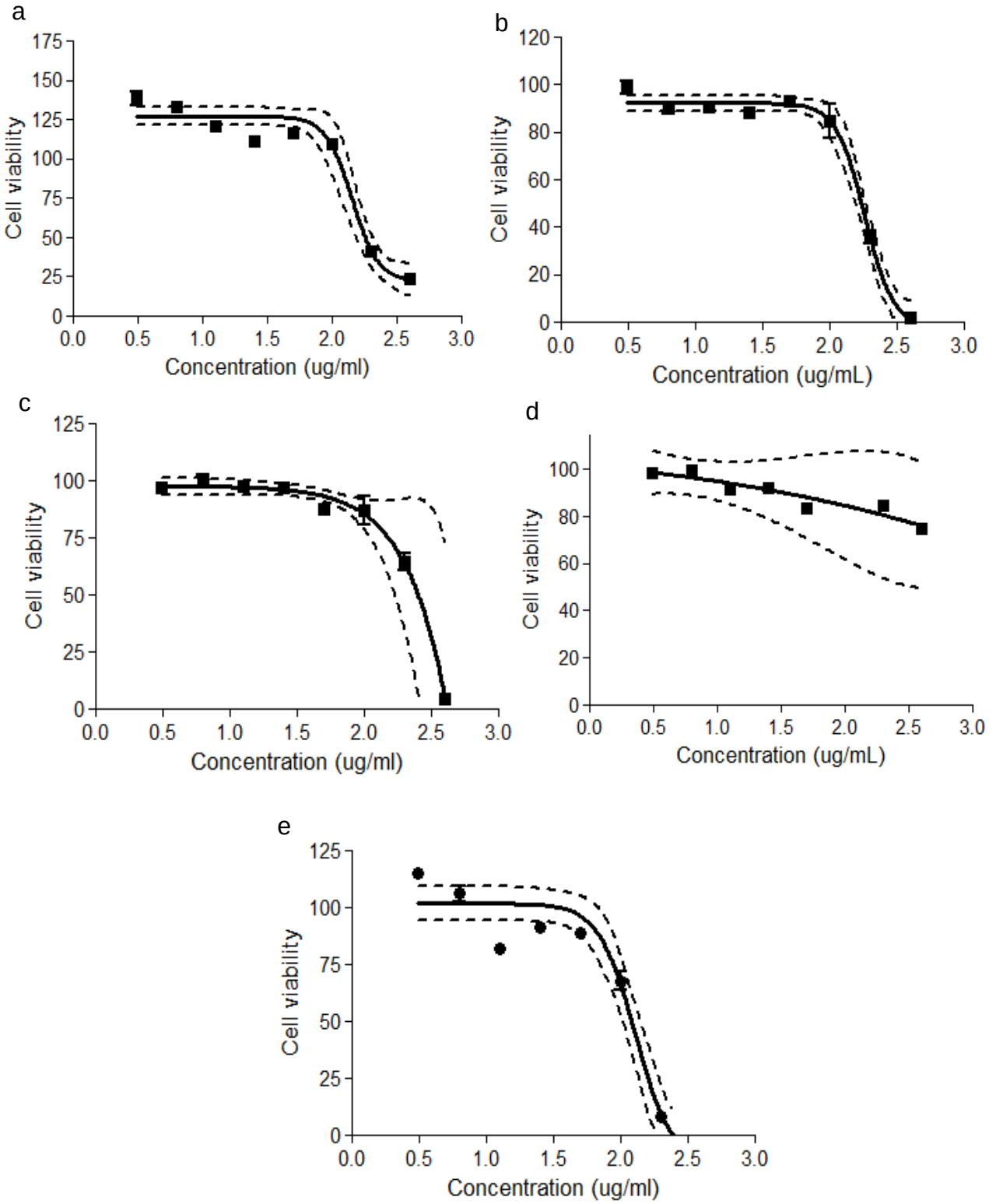

Figure 8.2: Cell viability graphs of selected plant extracts a) *E. lasianthum* ethanol, b) *H. natalensis* acetone, c) *H. natalensis* ethanol, d) *H. natalensis* water and e) *W. salutaris* acetone on the HepG2 cell line.

8.1 Gas chromatography-Mass spectrometry methodology

The method given below was used for the analysis of compounds present in sub-fraction 201-250. Dr. Yvette Naude at the Department of Chemistry, at the University of Pretoria, Pretoria, South Africa, used this method. The sub-fraction 201-250 was dissolve in methanol to obtain a concentration of 1 mg/mL. Analysis of 1 mg/mL of sub-fraction 201-250 was done using LECO Pegasus 4D GC-TOFMS (LECO Africa (Pty) Ltd., Kempton Park, South Africa). The GC capillary column used, was Rxi-1MS 30 m x 0.25 mm ID x 0.25 µm film thickness (Restek, Bellefonte, PA, USA). The injection volume used was 1 uL with a split injection of 10:1 GC inlet at 250 °C. The GC oven temperature programme was at 40 °C, held for 3 minutes and ramped at 8 °C/min to 300 °C then held for 5 min. The carrier gas, UHP Helium (Afrox, South Africa) was at constant flow mode for every 1 mL/min during which the Mass acquisition range was 40-500 Daltons. The Mass spectrometry transfer line and ion source temperature were maintained at 280 °C and 230 °C, respectively. The Mass spectrometry solvent delay was 5 minutes. The electron energy for the electron ionisation mode (EI+) was kept at 70 eV with the data acquisition rate and detector voltage at 10 spectra/second and 1750 V, respectively. The NIST14 Mass Spectral Library was used for Mass spectral identification.

Figure 8.3: The Gas chromatography-Mass spectrometry spectrum of compounds identified from the butanol fraction of *H. natalensis* (pooled fraction 201-250).

Table 8.2: Gas chromatography-Mass spectrometry of compounds identified from the butanol fraction of *H. natalensis* (pooled fraction 201-250).

Peak #	Name	Weight	1st Dimension Time (s)	Formula	Area %
11	Ethanedioic acid, dibutyl ester	202	130,1	$C_{10}H_{18}O_4$	23,77
10	n-Hexane	86	128,3	C_6H_{14}	22,512
13	Ethyl Acetate	88	134,6	$C_4H_8O_2$	9,0319
15	Ethyl Acetate	88	134,9	$C_4H_8O_2$	9,0319
14	Borane, ethyldimethyl-	70	134,7	$C_4H_{11}B$	9,0027
16	2H-Pyran-2-one, tetrahydro-6,6-dimethyl-	128	140,2	$C_7H_{12}O_2$	7,156
17	Ethyl Acetate	88	141	$C_4H_8O_2$	5,6156
92	Diisooctyl phthalate	390	1742,6	$C_{24}H_{38}O_4$	1,5539
47	Hexadecane	226	1109,1	$C_{16}H_{34}$	1,4712
54	Heneicosane	296	1272	$C_{21}H_{44}$	0,68963
66	4-(3,5-Di-tert-butyl-4-hydroxyphenyl)butyl acrylate	332	1525,7	$C_{21}H_{32}O_3$	0,64698
27	2,2-Dimethoxybutane	118	230,8	$C_6H_{14}O_2$	0,61579
41	Hexadecane	226	928,3	$C_{16}H_{34}$	0,4783
3	Acetaldehyde	44	116,3	C_2H_4O	0,47378
6	Ethanol	46	118,4	C_2H_6O	0,47378
5	Pentane, 2-methyl-	86	117,3	C_6H_{14}	0,47378
7	Benzeneethanamine, 2-fluoro-á,3-dihydroxy-N-methyl-	185	119,4	$C_9H_{12}FNO_2$	0,47378
4	3-[18-(3-Hydroxy-propyl)-3,3,7,12,17-pentamethyl-2,3,22,24-tetrahydro-porphin-2-yl]propan-1-ol	498	116,6	$C_{31}H_{38}N_4O_2$	0,47378
93	Sulfurous acid, isobutyl pentyl ester	208	1744,6	$C_9H_{20}O_3S$	0,45559

20	Cyclohexane	84	157	C_6H_{12}	0,36857
19	Propane, 2,2-dimethoxy-	104	155,8	$C_5H_{12}O_2$	0,36857
9	Isopropyl Alcohol	60	122,7	C_3H_8O	0,35403
8	Pentane, 3-methyl-	86	122,2	C_6H_{14}	0,35403
94	Unknown 3	310	1745,5	$C_{16}H_{17}F_3OSi$	0,35168
62	Heneicosane	296	1419,8	$C_{21}H_{44}$	0,28198
23	Triethylamine	101	189,7	$C_6H_{15}N$	0,17006
59	Benzenepropanoic acid, 3,5-bis(1,1-dimethylethyl)-4-hydroxy-, methyl ester	292	1352,8	$C_{18}H_{28}O_3$	0,13833
72	1H-Pyrazino[3,2,1-jk]carbazole, 2,3,3a,4,5,6-hexahydro-8-methyl-3-[2-(1-piperidinyl)acetyl]-	351	1646,5	$C_{22}H_{29}N_3O$	0,13751
25	Butanoic acid, methyl ester	102	198,8	$C_5H_{10}O_2$	0,11341
33	1-Pentene, 2-methoxy-	100	310,8	$C_6H_{12}O$	0,11105
43	Pentadecane	212	1021,1	$C_{15}H_{32}$	0,1009
104	Pentadecane	212	1887,7	$C_{15}H_{32}$	0,099337
34	1,3,5-Pentanetriol, 3-methyl-	134	314,1	$C_6H_{14}O_3$	0,092229
102	Sulfurous acid, octadecyl pentyl ester	404	1852,5	$C_{23}H_{48}O_3S$	0,090371
108	Benzenamine, 4-octyl-N-(4-octylphenyl)-	393	1979,4	$C_{28}H_{43}N$	0,081388
107	4-Nitrophenyl caprylate	265	1979	$C_{14}H_{19}NO_4$	0,081014
2	Pentaborane(11)	66	115,1	B_5H_{11}	0,080685
67	Heptacosane	380	1554,9	$C_{27}H_{56}$	0,080594
1	Methyl 2-butynoate	98	114,2	$C_5H_6O_2$	0,079319
99	Sulfurous acid, butyl heptadecyl ester	376	1799	$C_{21}H_{44}O_3S$	0,078768
35	2-Propanol, 1-(2-butoxy-1-methylethoxy)-	190	317,1	$C_{10}H_{22}O_3$	0,078696
73	2-Amino-5-isopropyl-8-methyl-1-azulenecarbonitrile	224	1650,4	$C_{15}H_{16}N_2$	0,077397

60	Dibutyl phthalate	278	1362,3	$C_{16}H_{22}O_4$	0,07735
49	Eicosane	282	1192,6	$C_{20}H_{42}$	0,075621
36	1,3-Dioxolane-4-methanol, 2-ethyl-	132	320,6	$C_6H_{12}O_3$	0,070724
53	Cetene	224	1264,7	$C_{16}H_{32}$	0,069595
46	Cetene	224	1100,3	$C_{16}H_{32}$	0,06782
40	Decane	142	496	$C_{10}H_{22}$	0,064211
71	Tert-octyldiphenylamine	281	1644,2	$C_{20}H_{27}N$	0,061853
39	Nonane	128	378,7	C_9H_{20}	0,046842
44	Pentadecane, 7-methyl-	226	1063,3	$C_{16}H_{34}$	0,046124
75	1-Ethyl-4-m-tolyl-1,4-dihydro-pyridine-3,5-dicarboxylic acid dimethyl ester	315	1656	$C_{18}H_{21}NO_4$	0,044922
96	Sulfurous acid, decyl pentyl ester	292	1780,1	$C_{15}H_{32}O_3S$	0,044492
42	2,4-Di-tert-butylphenol	206	1013,4	$C_{14}H_{22}O$	0,042765
84	Heptacosane	380	1679,2	$C_{27}H_{56}$	0,041252
112	1-Hydroxy-3-(octanoyloxy)propan-2-yl decanoate	372	2137,4	$C_{21}H_{40}O_5$	0,040929
32	1,3,3-Trimethoxybutane	148	291,5	$C_7H_{16}O_3$	0,040169
76	Unknown 1	344	1656,5	$C_{19}H_{20}O_6$	0,039593
98	Heptadecane, 2-methyl-	254	1793,7	$C_{18}H_{38}$	0,039101
95	Unknown 4	312	1758,6	$C_{22}H_{16}O_2$	0,038304
61	1-Docosene	308	1413,3	$C_{22}H_{44}$	0,038076
101	Hentriacontane	436	1848,4	$C_{31}H_{64}$	0,035595
22	3,5-Dithiahexanol 5,5-dioxide	170	174,8	$C_4H_{10}O_3S_2$	0,035251
18	Silane, dimethoxydimethyl-	120	153,1	$C_4H_{12}O_2Si$	0,034461
57	7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione	276	1330,5	$C_{17}H_{24}O_3$	0,034283
64	8,11,14-Eicosatrienoic	306	1470,3	$C_{20}H_{34}O_2$	0,034009

	acid, (Z,Z,Z)-				
51	2-Mercapto-4,5-dimethylthiazole	145	1227,3	$C_5H_7NS_2$	0,032673
58	Heptacosane	380	1347,8	$C_{27}H_{56}$	0,031813
106	4-tert-Octylphenol, TMS derivative	278	1910,7	$C_{17}H_{30}OSi$	0,031175
12	Trichloromethane	118	132,6	$CHCl_3$	0,027126
86	1-Iodo-2-methylundecane	296	1685,4	$C_{12}H_{25}I$	0,026623
100	Sulfurous acid, butyl dodecyl ester	306	1813,1	$C_{16}H_{34}O_3S$	0,02606
50	Heptadecane, 2,6-dimethyl-	268	1200,3	$C_{19}H_{40}$	0,02511
105	Unknown 6	470	1894,3	$C_{30}H_{46}O_2S$	0,024621
24	Silane, trimethoxymethyl-	136	196,7	$C_4H_{12}O_3Si$	0,024407
87	Heptacosane	380	1701	$C_{27}H_{56}$	0,023109
69	1-Iodo-2-methylundecane	296	1618,2	$C_{12}H_{25}I$	0,022886
56	Octadecane, 4-methyl-	268	1316,7	$C_{19}H_{40}$	0,022873
88	2,6-Di-tert-butyl-4-(3-mercaptopropyl)phenol	280	1714,5	$C_{17}H_{28}OS$	0,022165
90	1-Iodo-2-methylundecane	296	1729,4	$C_{12}H_{25}I$	0,021658
65	Eicosane	282	1488,8	$C_{20}H_{42}$	0,020547
79	3,6-Bis(N-methylamino)carbazole	225	1663,9	$C_{14}H_{15}N_3$	0,020231
80	Pyrido[2,3-g]indole-8-carboxylic acid, 9-hydroxy-2,3,5-trimethyl-, ethyl ester	298	1665,3	$C_{17}H_{18}N_2O_3$	0,020231
68	Tert-octyldiphenylamine	281	1584,2	$C_{20}H_{27}N$	0,019534
97	Heneicosane	296	1785,5	$C_{21}H_{44}$	0,019365
48	Hexadecane, 4-methyl-	240	1158,3	$C_{17}H_{36}$	0,018306
29	Butanoic acid, 2-methyl-, methyl ester	116	241,6	$C_6H_{12}O_2$	0,018277
91	Octacosane	394	1737,6	$C_{28}H_{58}$	0,017908
83	3,5-Dimethoxy-4-hydroxycinnamic acid	224	1676,6	$C_{11}H_{12}O_5$	0,016831

82	Tert-octyldiphenylamine	281	1674,8	$C_{20}H_{27}N$	0,016831
26	Silane, trimethoxymethyl-	136	202,1	$C_4H_{12}O_3Si$	0,015351
74	Tert-octyldiphenylamine	281	1653	$C_{20}H_{27}N$	0,01526
45	Adipic acid, di(2-methylpent-3-yl) ester	314	1077,2	$C_{18}H_{34}O_4$	0,010656
30	Tetramethyl silicate	152	244,3	$C_4H_{12}O_4Si$	0,0093837
85	Dimethyl 4-(2-bromophenyl)-1,4-dihydro-2,6-dimethylpyridine-3,5-dicarboxylate	379	1682,8	$C_{17}H_{18}BrNO_4$	0,0080309
31	Propane, 1,1-dimethoxy-2-methyl-	118	247,9	$C_6H_{14}O_2$	0,0067816
28	Furan, 2-butyltetrahydro-	128	238	$C_8H_{16}O$	0,006552
52	Isoquinoline, 1-butyl-3,4-dihydro-	187	1230,9	$C_{13}H_{17}N$	0,0063306
70	Naphthalene-2-carbonitrile, 3,4-dihydro-1-amino-3-spirocyclohexane-	238	1634,6	$C_{16}H_{18}N_2$	0,0059618
63	Sulfurous acid, cyclohexylmethyl hexadecyl ester	402	1435,9	$C_{23}H_{46}O_3S$	0,0046478
21	2-Furanmethanol, tetrahydro-	102	164,6	$C_5H_{10}O_2$	0,0043597
38	3-Methylbenzyl alcohol, TBDMS derivative	236	339,1	$C_{14}H_{24}OSi$	0,0034159
89	Phthalic acid, 2,2-dimethylpent-3-yl tetradecyl ester	460	1716,3	$C_{29}H_{48}O_4$	0,0031131
81	2-(1-(2-Methylpropyl)-3-methylbutyl)-5-phenylpyridine)	281	1670,1	$C_{20}H_{27}N$	0,0029146
103	Unknown 5	336	1860,6	$C_{19}H_{28}O_5$	0,0029113
55	Phthalic acid, hex-2-yn-4-yl hexyl ester	330	1293,6	$C_{20}H_{26}O_4$	0,0027258
78	Unknown 2	238	1661,5	$C_{16}H_{18}N_2$	0,0022604
77	Pregnenolone acetate	358	1659,9	$C_{23}H_{34}O_3$	0,0019838
111	Unknown 9	350	2063,8	$C_{26}H_{38}$	0,0019667

37	3-Methylbenzyl alcohol, TBDMS derivative	236	334,6	$C_{14}H_{24}OSi$	0,00083692
110	Unknown 8	355	2014	$C_{18}H_{17}N_3O_5$	0,00042112
109	Unknown 7	297	2011,6	$C_{18}H_{23}N_3O$	0