APPLICATION OF SOME TARGET FORMULATIONS OF ACTIVE HERBAL PLANT COMPONENTS IN REDUCING ANIMAL EXPOSURE TO MYCOTOXINS AND ASSOCIATED HEALTH EFFECTS

A Dissertation Submitted to the Faculty of Science, University of Johannesburg

In Fulfilment

for the requirement of a Masters of Technology in Biotechnology

By

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August 2015

EXECUTIVE SUMMARY

The presence of mycotoxigenic fungi and mycotoxins in food and feed commodities causes adverse health effects on both animals and humans. Herbal plant components remain an untapped reservoir for active compounds (phytochemicals) with properties that can potentially reduce the effects associated with animal exposure to mycotoxins. This is mainly through targeting the prevention of fungal crop infestation by screening for antifungal plant components and those that enable the reduction of mycotoxin-induced oxidative stress via antioxidant activity. Thus, the study reported herein evaluated the potential application of *Mentha longifolia, Leonotis leonurus* and *Piptadeniastrium africanum* plant extracts for reducing animal exposure to mycotoxins and associated adverse effects.

Sequential solvent extraction using hexane, dichloromethane, ethyl acetate and methanol was applied in the extraction of crude extracts from each of the three dried powdered plant materials. The highest % extraction yields were obtained using methanol as extracting solvent, which confirmed the presence of more polar than non-polar components in each plant material. The phytochemistries of the extracts were evaluated using thin layer chromatography (TLC), ultraviolet-visible spectroscopy (UV-Vis), Fourier Transform Infrared spectroscopy (FT-IR), and 2 dimensional time of flight mass spectroscopy gas chromatography (2D GCxGC-TOF/MS). The UV-Vis confirmed the presence of phenolic compounds such as tannins, polyphenols, quinones, phenolic acids and their derivatives. These were seen as FT-IR peaks with functional groups bond/group frequencies of phenolic acids and their derivatives at 3411.66, 3411.5, and 3314.92 cm^{-1}.

Amongst the compounds identified by GCxGC-TOF/MS in each plant extract, 6 were selected on the basis of either one or more of the pharmacological properties, i.e., antioxidant, anti-inflammatory, anticancer, antimicrobial and antifungal activity. They are á-pinene, I-menthone, apocynin, naphthalene, 1, 2, 3, 5, 6, 8a, hexahydro-4, 7-dimethyl-1(1-methylethyl), (1Scis), camphene, and à-terpineol from *M. longifolia*. 1-methyl-pyrrolidine-2-carboxylic acid, 5-hydroxypipecolic acid, cinnamaldehyde, (E)-, 2, 4-dihydroxy-2, 5-dimethyl-3(2H)-furan-3-one, 2-caren-10-al, and 2H-pyran-2, 6(3H)-dione from *L. leonurus*. The 6 compounds selected from the *P. africanum*

extract were resorcinol, N, N-dimethylglycine, indole, phenol, 2, 6-dimethoxy-4, (2-propenyl)-, vanillin, and pantolactone. In addition, phytol, n-hexadeconoic, 1, 2, 3 4 H-pyran-4-one, 2, 3-dihydro-3, 5-dihydroxy-6-methyl-, 3, 7, 11, 15-tetramethyl-2-hexadecen-1-ol were amongst the 12 compounds that co-occurred in either two or three of the plant extracts with the above-mentioned properties.

Antifungal activity screening was conducted using agar diffusion and microplate dilution assays. *Piptadeniastrium africanum* had a high antifungal activity with a zone of inhibition of 25 mm (1.56 % minimum inhibition zone) against *Aspergillus niger* on Day 3. The minimum inhibition concentration (MIC) of *P. africanum* was 2 mg/ml against *A. fumigatus*, *A. ochraceus*, and *A. niger*. The 2D GCxGC-TOF/MS data confirmed the presence of the fungicidal compound furfural at peak 27, with a yield of 0.31% in the methanol extract of *P. africanum*, which can be linked to the observed antifungal activity exhibited by the plant material.

The *in vitro* and *ex vivo* antioxidant activity of the extracts were measured using electron-paramagnetic resonance (EPR) spectroscopy. Generally, results indicated a concentration depended antioxidant activity seen in all the three plant extracts using 2, 2-diphenyl-1-picrylhydrazyl (DPPH) assay. Although, *P. africanum* expressed the most superior activity of 99% compared to that of *M. longifolia* and *L. leonurus,* whose activity were 17 and 28%, respectively, at 0.1%/10 µl. Exposure of all the three extracts to UV irradiation resulted in an increase in antioxidant activity. The *ex vivo* studies determined the superoxide dismutase (SOD), Ascorbyl (•Asc) radicals and reactive oxygen species (ROS) production levels in organ homogenates of mice administered with *P. africanum* extract. The SOD-like activity was at 12.22 Units/mg protein but no activity was recorded after exposure to UV irradiation. The average •Asc radical production levels in the liver were at 0.6141 and 0.3375, meanwhile brain homogenates recorded a mean of 0.6141 and 0.18305, for the sample and control, respectively. Relatively high levels of ROS radicals were recorded in the liver (1.0701 against 0.6699 for the control) and brain homogenates (1.7325 against 0.3167 for the control). It therefore shows that *P. africanum* exhibited *in vitro* and *in vivo* antioxidant and pro-oxidant activity.

The cytotoxicity and cytoprotection of the extracts was also evaluated using the methyl thiazol tetrazolium (MTT) assay. Data obtained indicated some isolated cases

of inconsistent increase in % cell viability in the presence of the mycotoxins, ochratoxin A (OTA) and fumonisin B_1 (FB_1) with no confirmed cytoprotection activity. The least cytotoxic extract among the three extract was *L. leonurus* exposed to cells at 0.1 mg/ml concentration for 24 hrs, meanwhile the extracts from *M. longifolia* and *P. africanum* were slightly more cytotoxic.

In conclusion, antifungal and antioxidant activity properties of the plant species of interest were impressive particularly in the case of *P. africanum*. Despite this, the *ex-vivo* studies suggest that *P. africanum* can act as a pro-oxidant with high levels of •Asc and ROS production. Thus, it is potentially not good for inclusion in animal feed. The weak antioxidant activity observed on *M. longifolia* and its slight cytotoxic activity also suggest that this plant is potentially undesirable for herbal protection. Although, *L. leonurus* had moderate antioxidant activity and low cytotoxic activity, the studies conducted herein lack sufficient evidence for its approval as a feed additive. Further fractionation to exclude the toxic components in these plants could yield desired components for inclusion in animal feeds for reducing animal exposure to mycotoxins and associated health effects. Therefore, there is need for further investigations to assess the efficacy of plant materials in providing some herbal protection.

Key words: Mycotoxins, oxidative stress, antioxidants, antifungal, herbal plants and phytochemicals.

PREFACE

I Makhosazana Lindiwe Dlamini hereby declare that this dissertation, I herewith submit for the qualification of a Masters of Technology degree in Biotechnology, to the University of Johannesburg, Faculty of Science, Department of Biotechnology, apart from the recognized assistance received from my supervisor Dr. P.B. Njobeh and co-supervisor Prof. R. Krause, is my work and has not been previously submitted by me for a degree qualification in another institution.

Signature: _____ **Date:**

DEDICATION

To the one and only God Almighty, my dearest mother Nomvula Maduna and my father Richard Dlamini, my sister Nomcebo, my brother Thando, boNguni bami and Sandzi who have been my comfort, I dedicate this work.

ACKNOWLEDGEMENTS

First and foremost my appreciation goes to God. To my supervisor Dr. P.B. Njobeh and co-supervisor Prof. R. Krause who have assisted every step of the progress and accomplishment of this project. Thank you for your mentorship. A special appreciation to Dr. P.B. Njobeh my supervisor for the student assistantship NRF-supervisor linked bursary and to Mr. E. van Zyl, the HOD of the Biotechnology Department, Faculty of Science, University of Johannesburg, for the award of a merit bursary.

This study was partly sponsored by the *Seventh Framework Programme of the European Union* SP3 – People, Support for training and carear development of researches (Marie Curie), International Research Staff Exchange Scheme (IRSES), FP7-PEOPLE-2012-IRSES, Grant Agreement Number 316067, on "Studies on some herbal additives giving partial protection against toxic or immunosuppressive effects of some mycotoxins and improving wound granulation"

To Dr. Stephen Musyoka, thank you for your assistance in the laboratory. Prof. Gajeva, Prof. Zheleva A., Dr. Nikolova G., a special appreciation for the EPR studies conducted in Trakia University, Bulgaria. To Dr. Ndinteh D. thank you for *Piptadeniastrium africanum* plant material and your guidance with the phytochemical studies and to Mr. Nyoni K. my appreciation for the GCxGC-TOF/MS technical support.

Thanks to Mrs. Karin Behr of the SANBI for allowing me to collect plant material at the Pretoria Botanical gardens, and Mr. Kangela Baloyi for assisting me in plant identification during the sampling process.

To my colleagues Mr. Fonkui T., Mr. Ngodjiga J. and Ms Phuko J., I sincerely appreciate the assistance you gave me throughout the project.

To my spiritual leaders the esteemed Pst. Goletlanwe A., Pst. Pasha K. and Pst. Maile I. my deepest gratitude for your investment and being spent on me.

To my family members: my parents Nomvula and Richard Dlamini, my aunt Ntombi, my siblings Nomcebo and Thando, beNgumi bami Lungisa, Phatsalive, and Sandziso. Thank you for your unconditional love. I love you more.

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LIST OF ACRONYMS AND ABBREVIATIONS

ABTS:	2, 2'-azinobis-3-ethylbenzothiazoline-6-sulfonicacid
AF:	Aflatoxin
AFs:	Aflatoxins
AREs:	Antioxidant regulatory elements
ATP:	Adenosine triphosphate
BEN:	Balkan Endemic Nephropathy
BHA:	Butylated hydroxyanisole
BHT:	Butylated hydroxytoluene
CAC:	Codex Alimentarius Commission
CAST:	Council for Agricultural Science and Technology
CAT:	Catalase
CCFAC:	Codex Committee on Food Additives and Contaminants
CCM:	Complete culture media
CIT:	Citrinin
CPA:	Cyclopiazonic acid
CO2:	Carbon dioxide
DMSO:	Dimethyl sulfoxide
DNA:	Deoxyribonucleic acid
DON:	Deoxynivalenol
DPPH:	2, 2-diphenyl-1-picrylhydrazyl
EDTA:	Ethylenediaminetetraacetic acid
EFSA:	European Food Safety Authority
EHC:	Environmental Health Criteria
ELEM:	Leukoencephalomalacia
EPR:	Electron paramagnetic resonance
ERK:	Extracellular signal-regulated protein kinase
FB:	Fumonisin
FB_1:	Fumonisin B_1
FB_2:	Fumonisin B_2
FB_3:	Fumonisin B_3
FBS:	Fecal bovine serum
FBs:	Fumonisins
FT-IR:	Fourier-transform infrared spectroscopy
FRAP:	Ferric decreasing antioxidant property
G0/G1:	G0: resting / G1: cell growth phases
GAP:	Good agricultural practices
GCxGC-TOF/MS:	Gas chromatography time of flight mass spectrometry

GMP:	Good manufacturing practices
GPx:	Glutathione peroxidase
HACCPs:	Hazard analysis and critical control points
HASCAS:	Hydrated sodium and calcium aluminosilicate
HO:	Hydroxyl
IARC:	International Agency for Research on Cancer
IITA:	International Institute for Tropical Agriculture
JNK:	Jun N-terminal kinase
LPOs:	Lipoxygenases
MAPK:	Mitogen-activated protein kinase
MDA:	Malondialdehyde
MIC:	Minimum inhibition concentration
MIZ:	Minimum inhibition zone
MN:	Mycotoxic nephropathy
MON:	Moniliformin
MRPs:	Maillard reaction products
MS:	Mass spectrometry
MTT:	Methyl Thiazol Tetrazolium
MZ:	Mass-to-charge ratio
NCI:	National Institude for Cancer
NMR:	Nuclear magnetic resonance
NO:	Nitric oxide
Nrf2:	Nuclear erythriod 2-related factor
NTB:	Nitro blue tetrasole
NTP:	National Toxicology Program
O_2^-:	Superoxide peroxyl
OD:	Optical density
OTA:	Ochratoxin A
OTB:	Ochratoxin B
OTC:	Ochratoxin C
OTs:	Ochratoxins
OTα:	Ochratoxinα
OTβ:	Ochratoxin β
PA:	Penicillic acid
PBN:	N-tert-butyl-alphaphenylnitrone
PBS:	Phosphate buffered saline
PHS:	Prostaglandin H synthase
PN/CN:	Porcine/chicken nephropathy
PROMEC:	Programme on Mycotoxins and Experinmental Carcinogenesis
PUFAs:	Polyunsaturated fatty acids
R_f:	Retardation factor
RNA:	Ribonucleic acid
RO:	Nucleophile
ROO:	Alkoxyl

ROO:	Peroxyl radical
ROOH:	Hydroperoxide
ROS:	Reactive oxygen species
RPMI:	Roswell Park Memorial Institute medium
Sa:	Sphinganine
SCF:	Scientific Committee for Food
So:	Sphingosine
SOD:	Superoxide dismutase
TBHQ:	Tert-butylhydroquinone
THD:	Hydroxycinnamic derivatives
TIC:	Total ion chromatogram
TLC:	Thin layer chromatography
TOP:	Target organ protection
tRNA:	Transfer ribonucleic acid
USA:	United States of America
UUC:	Upper urothelial cancer
UV:	Ultraviolet
UV-Vis:	Ultraviolet-visible spectroscopy
ZEN:	Zearalenone

LIST OF UNITS

%:	Percentage
µl:	Micro-liters
^{0}C:	Degrees Celsius
G:	Gauss (magnetic unit)
g:	Grams
hrs:	Hours
kg:	Kilograms
mg:	Milligrams
min:	Minutes
mm:	Millimeters
mW:	Microwave
nm:	Nanometers.
ppm:	Parts per million
s:	Seconds
U/gHb:	Units per gram haemoglobin
U/mg:	Units per microgram
U/mg protein:	Units per mg total protein enzyme

CHAPTER ONE

1. GENERAL INTRODUCTION

1.1 Background

This study primarily focused on the potential use of herbal plant components as feed additives, ultimately to reduce animal exposure to mycotoxins and their adverse health effects. Mycotoxins are described as metabolites produced during secondary metabolism in toxigenic fungi (Degirmoncioğlu *et al*., 2005). They are problematic contaminants in a variety of agricultural products (Pittet, 1998; CAST, 2003). Therefore, they are considered hazardous agents in foods and feeds (Malagutti *et al*., 2005; Viljoen, 2008) due to their ability to weaken animal health and productivity (D'Mello and Macdonald, 1998). These effects in monetary terms, account for millions of US dollars in loses (Zain, 2011).

Addressing the problem of mycotoxins in feed and food commodities has been man's quest since the mycotoxin gold rush that began in 1962 when about 100,000 turkey poults died in the United Kingdom after consuming a peanut meal contaminated with aflatoxins (Bennett and Klich, 2003; Zain, 2011). Since then, numerous studies around the world have proposed technological applications to facilitate the reduction of animal exposure to mycotoxins. Some of the detoxification and decontamination approaches considered so far include plant selection for fungal and mycotoxin resistance and the use of synthetic mycotoxin binders (CAST, 2003; Abdel-Wahhab and Kolif, 2008; CAC/RCP, 2003 rev. 2014) which could be physical, biological, and chemical in nature. The applications of these measures are however limited as fungi and mycotoxins still persist in various commodities. Therefore, there is still a need for continuous search for suitable alternative means to address these problems and enable them for applicability on a large scale.

The significance of mycotoxin contamination lies in the stability of these toxins and therefore their ability to resist most food processing applications (Tournas *et al*., 2001; Bullerman and Bianchini, 2007) and thus being carried along the food chain. This explains the presence of mycotoxins in edible animal derived products such as meat, milk, butter, cheese and eggs (CAST, 2003). The major concern is the

increased incidence of human and animal diseases directly or indirectly linked to ingestion of food and feedstuffs contaminated with mycotoxins (Peraica *et al*., 1999).

The mycotoxins of interest in the study are those that are problematic and have been implicated in causing spontaneous porcine/chicken nephropathy (PN/CN). Recently, studies conducted both in South Africa and in Bulgaria have attributed PN/CN to the consumption of cereal-based feeds (Stoev *et al*., 1998; 2002b) contaminated with a mixture of mycotoxins, including ochratoxin A (OTA) and fumonisin B_1 (FB_1) (Stoev *et al*., 2009; 2010a; 2011). These particular mycotoxicosis outbreaks have resulted in high mortality among affected animals (Stoev *et al*., 2000b; Deshmukh *et al*., 2005); a major concern to the animal industry.

The occurrence of Balkan Endemic Nephropathy (BEN) exhibiting mainly kidney damage and failure among humans in the Balkan areas has also been reported to result from dietary human exposure to OTA and FB_1 (Stoev *et al*., 2010a). These Balkan states are also incidentally affected principally by PN/CN. Furthermore, the increased incidence of human esophageal cancer in South Africa (within the Transkei region) has been associated particularly with increased FB_1 exposure levels via consumption of highly contaminated maize with the toxin (Sydenham *et al*., 1990). These toxic tendencies exhibited by these mycotoxins demonstrate the need to address the mycotoxin problem as a food safety concern.

1.2 Justification

Herbal plants remain the most untapped reservoir of potential therapeutic agents that can be exploited in reducing animal exposure to mycotoxins and associated health effects. Particularly, traditionally acclaimed medicinal plants should be investigated for their phytochemical properties. Some of these plants have an accredited history in ethno-veterinary medicine (Masika *et al*., 2000; Masika and Afolayan, 2002; van Wyk, 2013). Some possess significant immune stimulatory, hepatoprotective, anti-inflammatory, antifungal and antioxidant activities (van Wyk *et al*., 2009; Razavi *et al*., 2012). Thus, they have vital attributes as suitable candidates in developing anti-mycotoxin therapy feed additives.

Some herbal additives are classified as dietary interventions (Riley and Norred, 1999), having the potential to reduce mycotoxin toxicity *in vivo* (Scott, 1998; Amйzqueta *et al*., 2009). Artichoke extracts for example, were reported to have strong protective effects on the kidney, partially neutralizing some known toxic effects resulting from OTA exposure (Stoev *et al*., 2000a; 2002a). It also has strong antioxidant activity (Peréz-García *et al.*, 2000; Georgieva *et al*., 2012) and is capable of inducing the regeneration of liver cells due to the phenolic compounds it possesses (Wang *et al.*, 2003). However, there is a controversy surrounding the efficacy of phenolic compounds as having the ability to reduce the effects of mycotoxin-induced oxidative stress (Bolt and Stewart, 2012) as they generally effectively scavenge free radicals (Terry, 2001; Santacruz *et al*., 2012).

Oxidative stress is one of the mechanisms of mycotoxin toxicity as proposed by Scaaf *et al*. (2002), Marin-Kuan *et al*. (2006) and Sharma *et al*. (2013). This ultimately leads to lipid peroxidation and tissue damage (Surai *et al*., 2008; Omar, 2013) as seen in cases of kidney damage among affected animals as well as in BEN patients. In addition, the synergistic toxic effect of a combination of FB_1 and OTA is reported to be caused by the fact that these mycotoxins induce oxidative stress (Stoev *et al*., 2012) and increase lipid peroxidation (Surai *et al*., 2002; Marin-Kuan *et al*., 2006; Kumar *et al*., 2012). For example the nephrocarcinogenic effect of OTA has been linked to reduced antioxidant defense mechanism (Cavin *et al*., 2007) in affected animals. The question is whether the addition of herbal components with antioxidant activity in animal feeds can have some protection against the health effects caused by mycotoxins as suggested.

To address the question raised above, the following South African medicinal plants were selected for the study, i.e., *Mentha longifolia*, *Leonotis leonurus*, with the exception of *Piptadeniastrium africanum* which is a native of Cameroon. Research conducted on *Mentha longifolia* and *Leonotis leonurus* has shown their medical importance as anti-inflammatory agents (van Wyk *et al*., 2009). *Mentha longifolia* has been reported to act as a growth promoter, improve digestion, and inhibit intestinal pathogenic microorganisms in chickens (Al-Ankari *et al*., 2004). *Leonotis leonurus* has also been implemented in ethno-veterinary medicines used in the Cape regions of South Africa (van Wyk, 2013). *Piptadeniastrium africanum* has been shown to have significant antifungal activities against some mycotoxigenic fungal species

(Brusotti *et al*., 2013). The ethno-botanical approach to these herbal plants for research, employed in this study, offers strong indications about their biological activities (Cox and Balick, 1994). It is cost effective in terms of time and capital, when it is used as the basis for exploration of active components (George *et al*., 2001). The study also looked at using more of locally available indigenous plants that would make sourcing cost effective.

1.3 Aims and Objectives

1.3.1 Aims

In preparing this study, the aim was to determine the effectiveness of some target herbal plant components as antifungal agents against some mycotoxigenic fungi and as herbal protection components primarily as antioxidant additives for inclusion in animal feeds.

1.3.2 Objectives

To achieve the aim of the study (Section 1.3.1), the following objectives were met:

- To extract and characterize active biological components from some targeted medicinal plant materials;
- To test the antifungal activity of the plant extracts against some mycotoxigenic fungi;
- To determine the antioxidant activity of the plant extracts; and
- To test *in vitro* both cytotoxic and cytoprotective effects of the plant extracts against cultured human mononuclear lymphocyte cells exposed to OTA and FB_1.

CHAPTER TWO

2. LITERATURE REVIEW

2.1 INTRODUCTION

The chapter reviews literature firstly, on animal feeds, and feed contamination by fungi and their attendant mycotoxins. The mycotoxins, ochratoxin A (OTA) and fumonisin B_1 (FB_1) are discussed as the significant contaminants of focus in this study. The study then outlines some of the toxicological effects of these two mycotoxins in animals and humans. Particular interest is paid to the incidence of porcine/chicken nephropathy (PN/CN), Balkan endemic nephropathy (BEN), upper urothelial cancer (UUC), and esophageal cancer. Secondly, the chapter discusses oxidative stress as the hypothesized primary toxicity mechanism of mycotoxin action. The limitations of current mycotoxin decontamination and detoxification techniques are outlined, followed by herbal protection and why plants are a reservoir of potential antimycotoxin and antifungal therapeutic agents. Thirdly, the botanical description, distribution, phytochemistry, pharmacology and medical applications of some selected plant species are reviewed in this chapter.

2.2 Animal feeds

2.2.1 Defination and concepts

Animal feed contains all the necessary nutrients in the correct quantities and proportions sufficient for optimal growth and immunocompetency of animals (Butcher and Miles, 2002). These include different components, i.e., water and dry matter (carbohydrates, lipids, proteins, minerals and vitamins) (MacDonald *et al*., 2010). Even though, these feed components are essential for animal development and health and also cater for the growth of mycotoxigenic fungi in feeds, they will not be discussed in detail in this review.

Compounded feed is mainly composed of cereals, oilseeds, and their by-products (Makun *et al*., 2012). Concentrate feeds mainly consist of high energy and protein with low fiber components. Forage can be plants or plant parts other than separated grains fed to or to be grazed by domestic animals. It may be fresh, dry or ensiled (e.g., pasture, green chop, hay, haylage) (Saha *et al*., 2013). The review focuses on

the contaminations of either raw materials (cereals) or the packaged feeds mainly by fungi and their mycotoxins.

2.2.2 Feed contamination

Cereals constitute about 55% of the total dry matter in some animal diets (Malagutti *et al*., 2005). Therefore, it is one of the biggest contributors to both fungal and mycotoxin contamination in animal feeds, especially in South Africa (Viljoen, 2008) where like other countries in sub-Saharan Africa, there are favourable conditions for their development in these commodities. Alarming levels of mycotoxin contamination in compound feeds have been reported in South Africa (Njobeh *et al*., 2012), Korea (Kim *et al*., 2014) and 75-100% of feed samples tested in Europe were contaminated with multi-mycotoxins (Streit *et al*., 2012). The presence of fungi alone in feed means a decrease in the nutritional value of the feed (Zaki *et al*., 2012). This is estimated to cause a 5-10% reduction in animal performance (Golob, 2007), mainly due to feed refusal (Nelson *et al*., 1994). Thus, the presence of fungi and their secondary metabolites in cereal-based feeds is likely to impact negatively on both animal and human health; derailing global initiatives toward food safety and security. These aspects are discussed subsequently in this review.

2.2.2.1 Fungal infestations

There are several reports on the global contamination of feed/food commodities (Abdel-Wahhab and Kolif, 2008; Reddy *et al*., 2010). The culprit in most of these agro-economic crises are pathogenic (disease causing) and mycotoxigenic (mycotoxin producing) fungi or symbiotic (beneficial) endophytes. This is due to the development and spread of their spores as well as their secondary metabolites particularly, mycotoxins (D'Mello, 2001). Annually, at least 25% of crops globally are infested by fungi (Bryden, 2007), besides the 40% that is contaminated with fungal secondary metabolites (Pittet, 1998; Yiannikouris and Jouany, 2002; Lawlor and Lynch, 2005).

The fungal species of concern in this review are natural inhabitants of the soils on which crops are grown (Elmholt, 2008). Some act as parasites or saprophytes (Bhat *et al*., 2010), that when suitable conditions such as drought and heavy rains during

harvest in the field, invade and destroy crops. Others are symbiotic deriving their organic nutrients from living crop plants (Miller, 1995) but such fungi will not be reviewed herein but rather those considered significant in terms of their mycotoxin production ability. The most common and widespread mycotoxigenic fungi in foods and feeds are classified under the *Aspergillus*, *Penicillium*, *Fusarium*, *Stachybotrys* and *Claviceps* genera (Degirmoncioğlu *et al*., 2005; Marasas *et al*., 2008; Moretti *et al*., 2013). Other mycotoxigenic genera reported to occur in grains are: *Alternaria*, *Rhizopus*, *Mucor, Trichoderma, Paecilomyces, Chaetomium Cladosporium*, and *Acremonium* (Amadi and Adeniyi, 2009).

Fungal crop contamination can occur at the following stages: in the field, during harvest, storage and processing (Coulibaly *et al*., 2008; Reddy *et al*., 2010; CODEX, 2014). It has been reported that the classical predominant field fungi belong to the genera *Claviceps, Neotyphodium, Fusarium* and *Alternaria* (Surai *et al*., 2008). In the field, a plant can be infested by more than a single species of fungi, and each producing more than one type of mycotoxin (Viljoen, 2008; Zain, 2011). Storage fungi especially *Aspergillus, Penicillium, Rhizopus* and *Mucor* species that are predominant in grains after harvest also proliferate during storage (Amadi and Adeniyi, 2009). The factors that influence fungal infestations and mycotoxin production are discussed subsequently.

2.2.2.2 Factors that influence fungal infestation

The occurrence of fungi in various commodities either in the field or during storage is influenced by a number of eco-physiological factors. Such factors include nutrient availability, temperature, moisture, and insect infestation (Degirmoncioğlu *et al*., 2005; CAC/RCP, 2003 rev. 2014). These factors are governed by on-going fluctuations in climate worldwide. Climate shifts are influential to the diversity and quantity of mycotoxin producing fungi that occur in the environment. It also influences the susceptibility of host crops to insect and subsequently, fungal manifestations (Cotty and Jaime-Garcia, 2007).

The phenotypic and metabolic diversity of mycotoxigenic fungi greatly enables them to colonize a wide range of crops, as well as to adapt to a range of environmental conditions (Moretti *et al*., 2013). This is evident with the general observation that

fungi grow wherever organic material is available (Al-Fakih, 2014), provided that the environment is conducive. Mycotoxigenic fungal species are further distinguished according to their geographical distribution alongside the mycotoxins they produce. Species such as *A. flavus*, *A. parasiticus* and *A. ochraceus* are said to proliferate well under warm humid conditions. Thus, *Aspergillus* mycotoxins are predominant in the tropics and generally warm regions (D'Mello, 2004). *Fusarium* species are considered more ubiquitous, occurring as major cereal pathogens and known to cause head blight in wheat and barley as well as ear rot in maize (Zenedine *et al*., 2007), also proliferate exclusively in warm areas (D'Mello, 2004). *P. expansum* and *P. verrucosum* on their part, preferably occur in temperate environments (Miller, 1995). Regardless of whether the grain originates from temperate, sub-tropical or tropical climates, high humidity and rainfall during harvest increases chances of fungal infestations (Golob, 2007; Awad *et al*., 2010).

Fandohan *et al*. (2003) reviewed that at least 50-80% maize crops get contaminated with fungi if favorable conditions (ambient temperature and moisture) are met during drying and storage. These conditions are normal within subtropical and tropical regions (Afsah-Hejri *et al*., 2013), especially in the sub-Saharan African region. One would therefore expect significant levels of fungal contaminations in these areas. In addition, the traditional storage facilities currently being used to dry crops in these regions are often poorly constructed and inappropriate (Bennett and Klich, 2003; Viljoen, 2008; Njobeh *et al*., 2010; Stoev, 2013), and enable an increased susceptibility to mycotoxin contamination of these crops.

2.2.3 Mycotoxins

2.2.3.1 Definition and concepts

Mycotoxins are mostly described as nonproteinaceous, low-molecular weight compounds (Grenier *et al*., 2013) produced by filamentous aerobic fungi as organic secondary metabolites (Yu, 2004). They are produced through diverse biosynthesis pathways, thus they vary in their chemical structures and so their mechanism of toxicity (Girish and Smith, 2008). The term ‘mycotoxin’ was coined after the 1961 London veterinary crisis that left about 100,000 turkey poults dead. The mysterious aflatoxicosis outbreak resulted from ingestion of aflatoxin contaminated peanut meal

(Bennett and Klich, 2003; Zain, 2011; Makun *et al*., 2012). Since then, a number of outbreaks have occurred, a couple of them reported in Nelson *et al*. (1994), Bryden (2007), and Stoev (2013). All these incidences led to ongoing scientific research on a variety of fungal toxins that contaminate feed/food commodities.

2.2.3.2 Mycotoxin production

Mycotoxin production is a complex phenomenon that has not been fully clarified. Some have suggested that fungal biosynthetic regulation of sporulation mechanisms at genetic level is related to secondary metabolite production (Calvo *et al*., 2002; Yu and Keller, 2005). For instance, a study on fumonisin production by *Fusarium proliferatum* revealed the *FUM* cluster are responsible for the regulation of the entire fumonisin biosynthetic pathway with the key enzyme, polyketide synthase, encoded by the *FUM*1 gene (Stępień *et al*., 2011).

Others have explained mycotoxin production in fungi in clear simple terms as condition of secondary metabolism in eukaryotes. Primary metabolism in fungi, just like for most eukaryotes, essentially caters for growth by the use of nutrients to provide energy, synthetic intermediates and key macromolecules. Secondary metabolism ideally caters for extrinsic functions which impinge on growth and reproduction of competing organisms within the immediate environment (Magan and Aldred, 2007). In more specific terms, secondary metabolism in fungi has also been proposed to be a response to oxidative stress (Reverberi *et al*., 2010) caused by a number of factors including environmental and or physiological stress (Bhatnagar *et al*., 2008), microbial competitive interactions (Prange *et al*., 2005), host plant-mycotoxigenic fungus interactions (Cleveland *et al*., 2004) and ecological imbalance (Magan and Aldred, 2007).

2.2.3.3 Mycotoxin prevalence

There are currently more than 300 mycotoxins known to exist, and the number keeps increasing as new ones are being discovered (Pohland, 1993; Fink-Gremmels, 1999; Zaki *et al*., 2012). Yet, only about 30 of these have analytical techniques properly developed for routine analysis (Surai *et al*., 2008). A couple of them have biomarkers for their identification available (CAST, 2003). This may imply that consumers could

be exposed to a combination of undefined mycotoxins that could have synergistic or additive effects. Mainly because, some mycotoxins though they may be present at low concentrations or even below the detection limit (Kanora and Maes, 2009), they have the potential to cause more problems than a single mycotoxin at a higher dose (Surai *et al*., 2010; Mwanza *et al*., 2011; Njobeh *et al*., 2012).

Attention has been paid to those mycotoxins that are of health and agro-economic importance (Peraica and Dominjan, 2001; Surai *et al*., 2008; Zain, 2011). They are: aflatoxins (AFs), ochratoxins (OTs), trichothecenes, zearalenone (ZEN), femorgenic toxins, fumonisins (FBs), tremorgenic toxins, patulin, and ergot alkaloids (Golob, 2007; Reddy *et al*., 2010). This review focuses on ochratoxins and fumonisins as the main significant mycotoxins in animal feeds.

2.2.4 OCHRATOXINS (OTs)

2.2.4.1 Ochratoxins producing fungi

Ochratoxins are produced by certain fungi belonging to the genera *Aspergillus* and *Penicillium* (Rumora and Žanić-Grubišić, 2009) but the principal producers are *A. alliaceus, A. aviricomus, A. carbonarius, A. glaucus, A. melleus,* and *A. niger, A. ochraceus, A. carbonarius, A. sclerotiorum, A. sulphures, P. verrucossum, P. verrucosum* (Benford *et al*., 2001) and *P. viridicatum* (Abdel-Wahhab and Kolif, 2008). The more recent producers reported are *A. steynii* and *A. westerdijkiae* (Gil-Serna *et al*., 2011).

Penicillium verrucosum is usually the main ochratoxin A (OTA) producer in temperate areas of the world (Holmberg *et al*., 1991), whereas *A. ochraceus* commonly produces OTA in tropical regions (D'Mello, 2001). Studies have shown that cereal contamination with *P. verrucosum* occurs mostly during storage, especially when the grain is not properly dried (Kuiper-Goodman, 1996). In such a case, fungal growth begins around moistened areas, before it spreads throughout the grain (Viljoen, 2008).

2.2.4.2 Chemistry of ochratoxins

OTA was first isolated from a culture of *A. ochraceus* and characterized in South Africa (van der Merwe *et al*., 1965). Ochratoxins are comprised of a polyketide-derived 3, 4-dihydro-3-R-methyl-isocoumar moiety linked by an amide bond to L-β-phenylalanine (Stoev, 2008) at the 12-carboxyl group (Kuiper-Goodman and Scott, 1989). Its structure has been shown to consist of a dihydroisocoumarin moiety (the pentaketide-derived OTα), linked through the carboxyl group to phenylalanine (Størmer, 1992; Harris and Mantle, 2001; Bayman and Baker, 2006). The isocoumarin moiety is formed from acetate units via the pentaketide pathway; carboxylated, and then chlorinated by chloroperoxidase to form OTA (Petzinger and Weidenbach, 2002). The final step, the linkage through the carboxyl group to phenylalanine, is catalyzed by OTA synthetase. Ochratoxin A has corresponding des-chloro analogues known as ochratoxin B (OTB), ochratoxin C (OTC), ochratoxin α (OTα) and ochratoxin β (OTβ) (Khoury and Atoui, 2010; Wu *et al*., 2011). Ochratoxin B usually forms when chlorine concentrations are low, and to an extent by dechlorination of OTA (Petzinger and Weidenbach, 2002; Wu *et al*., 2011). Figure 2.1 below shows the structures of ochratoxins, OTA being the most prevalent and the focus of this study.

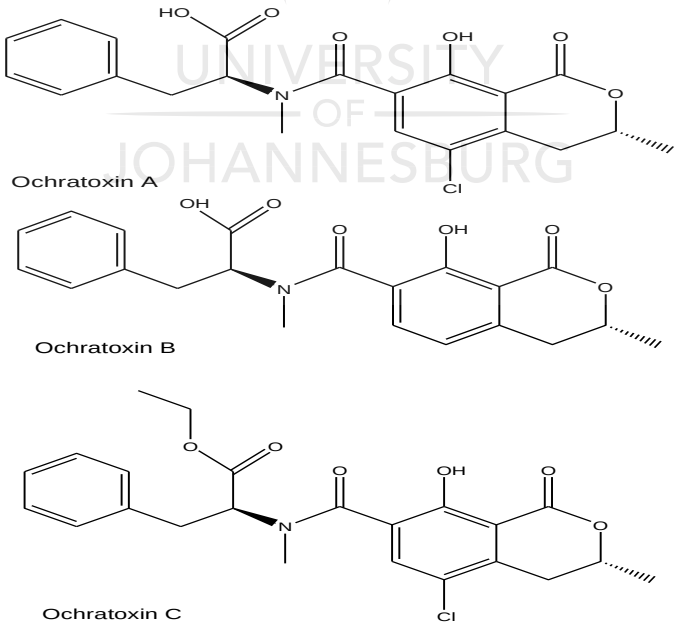


Figure 2.1: The chemical structures of ochratoxins A, B, and C

2.2.4.3 Occurrence of ochratoxins

Ochratoxin A (OTA) and OTB are the two forms that occur naturally as contaminants of crop products (Sahu and Satapathy, 2014), though, OTA is more ubiquitous, and occurs mostly in cereal grains and in the tissues of animals fed OTA contaminated feed (Council Directive 1999/29/EC rev. 2003). Pittet (1998), reported 25-40% OTA contaminations in cereal globally, more alarming levels of 40-50% OTA contaminations in unprocessed cereals were reviewed in Duarte *et al*. (2010). This is critical as cereal is a major constituent of animal feeds (Malagutti *et al*., 2005) as well as high rates of what humans consume (Khoury and Atoui, 2010). Marin *et al*. (2009) reviewed that 30µg/kg compound feed to be contaminated with OTA, thus cereal based feed is the main route of animal exposure to OTA (Duarte *et al*., 2011).

2.2.4.4 Ochratoxin A toxicity

OTA is nephrotoxic and hepatotoxic thus affecting mainly the kidney and liver (Beardall and Miller, 1994; Tsubouchi *et al*., 1995; Khoury and Atoui, 2010). In the case of nephrotoxicity, Kuiper-Goodman and Scott (1989) observed kidney lesions in animal models after OTA exposure. Changes in the renal function result in impairment of proximal tubular function, altered urinary excretion and increased excretion of urinary glucose (CAST, 2003; Khan, 2010; Thakur, 2010) mainly because of to the enterohepatic recirculation and the hepatobiliary way in which OTA is excreted (Stoev *et al*., 2002b; Koynarski *et al*., 2007; Rumora and Žanić-Grubišić, 2009).

The lactone group within OTA's structure is actively involved in its toxicity mechanism by covalently modifying enzymes (Xiao *et al*., 1996). This is evidently confirmed with the competitive inhibition of phenylalanine tRNA synthetase as one of the speculated toxicity mechanisms of OTA (Rumora and Žanić-Grubišić, 2009). Thus, the growth depression observed in affected animals has been linked to the disruption of protein synthesis (Beardall and Miller, 1994). In addition, the inhibition of the enzymes involved in phenylalanine metabolism observed in OTA affected animals (Creppy *et al*., 1990), has also been linked to the inhibition of mitochondrial ATP production and the stimulation of lipid peroxidation (Marquart *et al*., 1992). Other enzymes for example, succinate dehydrogenase and cytochrome C oxidase in rat mitochondria

were reported to be competitively inhibited by OTA as reviewed by Xiao *et al*. (1996). Also, cytosolic phosphoenolpyruvate carboxykinase was reported to be affected by OTA, leading to a decrease in gluconeogenesis (Rumora and Žanić-Grubišić, 2009).

Feeding broilers with OTA concentration ranging from 0.3-1 mg/kg was reported to cause reductions in glycogenolysis (Zain, 2011). This was attributed to a concentration dependent accumulation of glycogen in the liver because of the inhibition of cyclic adenosine 30, 50-monophosphate-dependent protein kinase as reviewed by Piotrowska *et al*. (2013). Thus, ultimately it decreases feed utilization efficiency and causes teratogenic malformations according to the review of Zain (2011). These effects are usually accompanied by dehydration, diarrhea, polyuria and polydipsia, and eventual death in pigs dosed with OTA (Battacone *et al*., 2010). In such vulnerable animals, growth depression is common (Varga *et al*., 2001; Malagutti *et al*., 2005). The vulnerability of pigs together with poultry is mainly because they are monogastrics, lacking the ability to rapidly degrade the toxin. Conversely, ruminants are able to hydrolyse the peptide bond of OTA into phenylalanine and a non-toxic OTα with the aid of symbiotic microbes present in the rumen (Malagutti *et al*., 2005). Ochratoxin A is potentially carcinogenic to humans (Group 2B carcinogen) (IARC, 1993; Bayman and Baker, 2006). An updated risk assessment of OTA placed greater emphasis on the low levels of OTA required to initiate aggressive tumour development and progression (Kuiper-Goodman, 1996; CCFAC, 1999). Therefore, there is high risk associated with exposure to OTA and possibly, other carcinogenic mycotoxins like fumonisins.

2.2.5 FUMONISINS

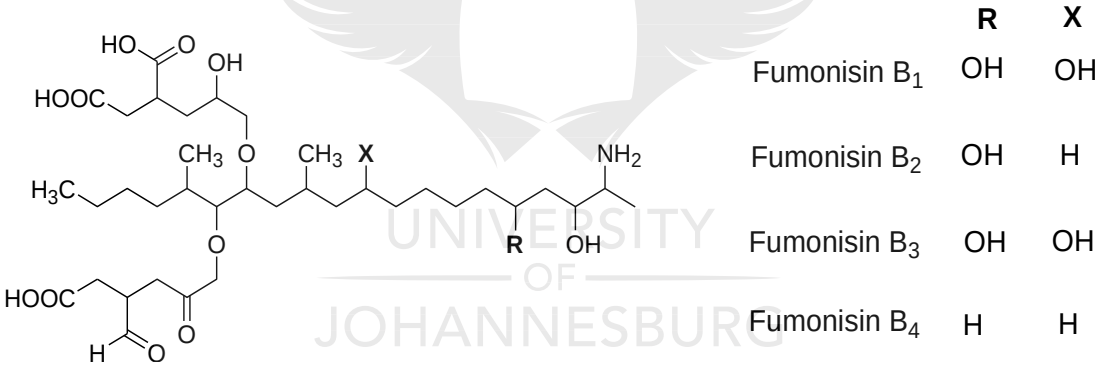
2.2.5.1 Fumonisin producing fungi

Fumonisins were first discovered and characterised in 1988 by a group of South African scientists working at the Programme on Mycotoxins and Experinmental Carcinogenesis (PROMEC) unit of the Medical Research Council (Bezuidenhout *et al*., 1988; Gelderblom *et al*., 1988). They are produced by *Fusarium* species such as *F. verticillioides, F. proliferatum, F. nygamai, F. lycopersici* as well as the species *Alternaria alternata f. sp. Lycopersici* (Rheeder *et al*., 2002)*. Fusarium napiforme, F. anthophilum,* and *F. dlamini* have also been considered as producers of fumonisins

(NTP, 1999; EHC, 2000). However, the principal agro-economic producers are *F. verticillioides* and *F. proliferatum* which frequently occur in maize (IARC, 2002) and maize-based feeds (Voss *et al*., 2007).

2.2.5.2 Chemistry and distribution of fumonisins

Fumonisin toxins are grouped into four main analogues, i.e., FB_1, FB_2, FB_3 and FB_4 (Nelson *et al*., 1994) with the first three being in order of occurrence, the common contaminants of maize globally (Miller, 2002; Devriendt *et al*., 2009). The biosynthesis of FB_1 has been described to begin with serine and palmitoyl-CoA forming 3-ketosphinganine. The 3-ketosphinganine is converted to sphinganine and again to sphingosine, which in turn can be converted to glycosphingolipids such as ceramide, which are further converted into sphingomyelin or other complex glycosphingolipids (SCF, 2000). Figure 2.2 shows the chemical structures of FB analogues.



	R	X
Fumonisin B_1	OH	OH
Fumonisin B_2	OH	H
Fumonisin B_3	OH	OH
Fumonisin B_4	H	H

Figure 2.2 The chemical structure of fumonisins B1, B2, B3, and B4

In terms of their distribution, alarming levels of up to 100% of these FB mycotoxins in maize grown by subsistence farmers has been reported in Centane region, located in the Eastern Cape of South Africa (Shephard *et al*., 2013). The most prevalent type reported being FB_1, contaminating 59% of maize and maize-based commodities (Devriendt *et al*., 2009).

2.2.5.3 FB_1 mechanism of toxicity

Fumonisin B_1 affects animals primarily by interfering with sphingolipid metabolism. This is because it is structurally similar to cellular sphingolipids. Thus, it is able to act

as a substrate, competitively inhibiting the enzyme ceramide synthase (Riley *et al*., 2001; Desai *et al*., 2002; Riley and Voss, 2006) during sphingolipid metabolism. This leads to an altered sphingolipid regulation and ultimately the increases in the levels of sphinganine (Sa) and sometimes sphingosine (So) in cells (Merrill *et al*., 2001; Stockmann-Juvala and Savolainen, 2008). The accumulation of Sa/So induced by exposure to FB_1 (Cai *et al*., 2007) has been reported to cause disturbances or alteration in cellular processes such as signal transduction pathways, cell growth, differentiation and morphology, endothelial cell permeability (SCF, 2000) and apoptosis in the liver and kidney cells (Voss *et al*., 2007) as well as mitosis (Desai *et al*., 2002; Devriendt *et al*., 2009). Despite this, Enongene *et al*. (2002) and van der Westhuizen *et al*. (2010) reported controversial data concerning the use of Sa/So levels as biomarkers in determining exposure to FB_1.

The fumonisin mycotoxins have been associated with a number of health effects in animals (D'Mello, 2000). Beri *et al*. (1991) reported changes in alkaline phosphatase, acid phosphatase, lactate dehydrogenase and succinate dehydrogenase in the heart, liver, spleen and pancreas of 1 week old chicks. Severe liver and lung damage as well as pancreatic lesions in pigs have also been reported due to FB_1 exposure (Diaz and Boermans, 1994). These disorders were said to be the cause of the observed overall reduced body weight gain and tissue necrosis (Beri *et al*., 1991; Zain, 2011) as well as biliary hyperplasia and thymic cortical atrophy (Ledoux *et al*., 1992) of the liver and kidney, thus supporting the view that FB_1 affects mainly these organs (Merrill *et al*., 1997).

Fumonisin B_1 also has negative effects on the gastro-intestinal tract (GIT) (Bouhet and Oswald, 2007) and the immune system (Voss *et al*., 2007). In pigs for example, the toxin is said to encourage intestinal invasion by pathogenic strains of *Escherichia coli* (Oswald *et al*., 2003) and *Salmonella* spp. (Deshmukh *et al*., 2005; Burel *et al*., 2013) as well as *Brucella* and *Listeria* (Girish and Smith, 2008). This effect is a result of immunosuppression and an altered intestinal barrier function due to altered sphingolipid metabolism. It is also demonstrated by the accumulation of glycolipids in the plasma membrane as well as a high trans-epithelial flux (Loiseau *et al*., 2007). Deshmukh *et al*. (2005) associated this with observed severe diarrhea and high mortality in affected animals. Amongst other immune-related defects observed in FB_1 exposed animals, is a decreased phagocytic potential of macrophages in chicks

reviewed by Voss *et al*. (2007), low cell viability and mitogenic response in splenic cells (Keck and Bodine, 2006), alteration of cytokine production (Girish and Smith, 2008), and decreased specific antibody responses (Taranu *et al*., 2005). All these findings support that the immunosuppressive effects caused by FB_1 exposure predisposes affected animals to secondary infections (Oswald *et al*., 2003).

One of the most profound effects linked to FB_1 exposure is the ability to induce resistant hepatocytes, a trait that is similar to many known hepatocarcinogens (Gelderblom *et al*., 1993), and may be the reason why FB_1 is potentially carcinogenic (IARC, 2003). The toxin has been correlated with the occurrence of esophageal cancer in humans (Marliere *et al*., 2009), particularly in the Transkei region of South Africa (Omar, 2013). Studies reviewed by Chelule *et al*. (2001) in this region, precisely in the Butterworth and Kentani districts, evidently correlated the high esophageal cancer incidence to the high intake of FB_1 contaminated maize. Maize-based ingredients are the main carbohydrate sources consumed almost daily in rural communities. These findings were compared to the low levels of FB_1 contamination in Lusikisiki and Bizana (districts within the Transkei region) (Sydenham *et al*., 1990) which displayed low esophageal cancer occurrences. These findings also correlated to those observed in other studies on the same subject conducted in the Linxian area in China (Rheeder *et al*., 1992; Chu and Li 1994; Yoshizawa *et al*., 1994) as well as in northeast of Italy (Doko and Visconti, 1994; Bhat *et al*., 1997).

Fumonisin exposures have been also associated with neural tube problems reported amongst underdeveloped communities living in Transkei and Kwa-Zulu Natal regions of South Africa and some parts of Northern China (Marasas *et al*., 2004; Milicevic *et al*., 2010). When humans and animals are exposed to multiple mycotoxins such as OTA, FB_1 and AFB_1, which is a common phenomenon for most of the populations, especially in rural settings, some synergistic and additive actions are exhibited. This is reviewed subsequently in this chapter.

2.2.6 Multi-mycotoxin aetiology

Mycotoxic nephropathy (MN) is a renal disease caused by the alimentary ingestion of mycotoxins with nephrotoxic properties (Stoev *et al*., 2002b). This disease has been described as chronic intestinal fibrosis, chronic intestinal nephritis or ochratoxicosis

(Stoev *et al*., 2009). It has been hypothesized that porcine/chicken nephropathy (PN/CN) is caused by a multi-mycotoxin aetiology involving OTA, FB_1 and penicillic acid (PA) or Citrinin (CIT) (Stoev *et al*., 2010a). This problematic mycotoxicosis is widely encountered in Bulgaria, Romania, Yugoslavia, Denmark (Petkova-Bocharova and Castegnaro, 1991; Stoev and Petkova-Bocharova, 1994), India (Shetty and Bhat, 1997; Jindal *et al*., 1999) and for the first time in South Africa, it was found to be prevalent in pig farms by a group of scientists working at the University of Johannesburg (Stoev *et al*., 2010b). Animal feeds from these farms with the observed nephropathy problem were found to be contaminated with FB_1 and OTA. In Bulgaria for example, FB_1 levels quantified from feed samples from affected farms ranged from 3000-5000 ppb and OTA levels ranged from 200-400 ppb (Stoev *et al*., 2010a). Meanwhile in South African feeds, FB_1 levels as high as 5000 ppb and OTA ranging from 60-70 ppb were reported in affected farms (Stoev *et al*., 2010b; Stoev *et al*., 2011). Comparable observations were made in various animal feeds from South Africa by Njobeh *et al*. (2012).

The low contamination levels of OTA in feed between 200 and 400 ppb, which may not induce any adverse health effects and the high incidence of PN and CN in Bulgaria have been the main reason for the hypothesized possible synergistic or additive interaction between OTA, FB_1 and possibly, other mycotoxins (Stoev *et al*., 2012; Stoev and Denev, 2013). These findings have been used to explain the observed enhancement of OTA nephrotoxicity, regardless of the lower levels of OTA found in feed samples (Stoev *et al*., 2011) which can easily be dismissed as having no potential to cause any noticeable effect in animals. The phenomenon of the occurrence of multi-mycotoxins-induced synergistic or additive effects (Njobeh *et al*., 2009) is not isolated to cases of PN/CN but occurs also with other mycotoxins and diseases as reviewed by Afsah-Hejri *et al*. (2013).

There are some speculations that Balkan endemic nephropathy (BEN) in humans and animals results from exposure to OTA and FB_1 (Radic *et al*., 1997; Stoev *et al*., 2009). The condition is a progressive chronic nephritis that occurs in populations living in the Balkan states including Romania, Bulgaria, Yugoslavia, Bosnia, Croatia, Romania and Serbia (Stoev, 1998). These areas are incidentally also mostly affected by PN/CN (Stoev *et al*., 2010a). Animals or humans with BEN suffer from or develop kidney damage that progresses to kidney failure. This condition is also coupled with a

bladder cancer called upper urothelial carcinoma (UUC) (Grollman *et al*., 2007) that eventually leads to death.

2.2.7 Plausible mycotoxin toxicty mechanism

2.2.7.1 Oxidative stress

Oxidation is the transfer of electrons from one atom to the other essential for cell metabolism with O_2 as an electron acceptor releasing energy in the form of ATP. It however, becomes problematic when electron flow becomes uncoupled causing the transfer of unpaired single electrons instead of paired ones, generating free radicals (Peréz and Aguilar, 2013). The generated reactive free radicals containing O_2 are known as reactive oxygen species (ROS), oxidants or pro-oxidants as reviewed by Gülçin (2012). They include hydroxyl (HO), superoxide (O_2^-) peroxyl (ROO), alkoxyl (RO) and nitric oxide (NO) (Nikolova, 2012). If ROS are not effectively scavenged by cellular constituents, they can stimulate oxidative stress (Halliwell and Gutteridge, 1990).

Packer (1995) stated that oxidative stress results from either a reduction in the antioxidant defense mechanism or an increase in ROS production. It occurs when the concentration of ROS generated exceeds the antioxidant capability of the cell (Guo *et al*., 2014). Under normal conditions, ROS are scavenged from cells by antioxidant enzymes i.e., superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) (Hou *et al*., 2013; Omar, 2013), heme oxygenase and biliverdin reductase (Murugan *et al*., 2012). Additionally, the most common chemical species that actively scavenge radicals are hydroxyl, sulfhydryl and amino groups (Atroshi *et al*., 2002). Oxidative stress and ROS can also be generated from xenobiotic bioactivation by prostaglandin H synthase (PHS) and lipoxygenases (LPOs) or microsomal P450s, which can be mycotoxin-induced (Omar, 2013). Mycotoxins have been implicated in the generation of ROS causing cellular damages in various animal species. This aspect is briefly discussed subsequently.

2.2.7.2 Oxidative stress biomarkers reported in mycotoxins

The mycotoxins AFB_1, FB_1, OTA, T-2 toxin and deoxynivalenol (DON) are known to cause cell membrane damage through oxidative stress induced lipid peroxidation

(Surai *et al*., 2002). The classical biomarkers of oxidative stress are identical to mycotoxin exposure biomarkers. These include lipid peroxidation, protein carbonyl, glutathione content, and antioxidant enzymes. More defined indicators reported by Kumar *et al*. (2012) are generation of ROS, activation of ERK1/2, p38 and JNK MAPKs, a 37-67% cell cycle arrest at the G0/G1 phase, a 2-10 fold increase in the induction of apoptosis, etc. These were reported as a result of the administration of OTA at 80 mg/mouse for a period of 12-72 hrs; thus, the emphasis that oxidative stress is one of the possible mechanisms of mycotoxin toxicity is eminent.

The synergistic tendency in generating cytotoxicity by FB_1 and OTA may perhaps be linked to the ability of these toxins to impair protein synthesis and enhance lipid peroxidation generating ROS (Stoev *et al*., 2009). Thus, feeding animals with diet supplemented with herbal additives containing antioxidants, could possibly provide some protection as suggested by Stoev *et al*. (2002a; 2004) and Creppy *et al*. (2004) or even the prospect of using phenolic antioxidants in inhibiting the biosynthesis of mycotoxins (Campbell *et al*., 2008) could confer such a protective action.

2.2.7.3 Oxidative stress-induced DNA adducts

It is also noteworthy to mention that a majority of studies have documented the formation of DNA adducts as biomarkers of oxidative stress induced by mycotoxins (Scaaf *et al*., 2002; Faucet *et al*., 2004; Kamp *et al*., 2005; Sharma *et al*., 2013). Studies reviewed by Arbillaga *et al*. (2008) on OTA toxicity have also indicated that the observed renal toxicity and DNA damage due to OTA exposure are attributable to oxidative stress (Mally *et al*., 2005; Marin-Kuan *et al*., 2006). Others have reported on the formation of DNA-adducts through a quinone/hydroquinone redox couple generated and attributed to OTA oxidative stress-induced effects (Xiao *et al*., 1995; Pfohl-Leszkowicz and Castegnoro, 2005; Tozlovanu *et al*., 2006); although a number of studies reviewed by Arbillaga *et al*. (2007) and Mosesso *et al*. (2008) disagree. Thus, the controversy on this subject and lack of clarification as to whether the plausible oxidative stress-induced mycotoxin theory is legible or not, exists. Furthermore, a likely association between oxidative DNA damage and OTA-induced mutations has equally been hypothesized (Palma *et al*., 2007).

2.2.7.4 Oxidative stress and carcinogenicity

OTA is most unlikely to act through a single, well defined mechanism of action, especially when it comes to carcinogenicity. Rather, it is proposed that a network of interacting epigenetic mechanisms, including oxidative stress and the activation of specific cell signaling pathways, may be responsible for OTA carcinogenicity as reviewed by Omar (2013). For example, a 2 year study in male F344 rats exposed to OTA (300 ug/kg bw) showed that OTA alters a sequence of genes in the kidney, which are regulated by nuclear erythriod 2-related factor (Nrf2) (Marin-Kuan *et al*., 2006).

Cavin *et al*. (2007) reported a profound link between OTA exposure and the induction of oxidative stress expressed by Nrf2-regulated genes. These genes regulate the enzymes responsible for cellular detoxification, cytoprotective, and antioxidant mechanisms in response to oxidative stress (Lee and Johnson, 2004; Kensler *et al*., 2007). The observed results indicated a correlation between OTA exposure and the downregulation of Nrf2 gene expression with an increase in oxidative stress and DNA-damage (Cavin *et al*., 2007; Kumar *et al*., 2012). Such a response has been associated with the development of kidney cancer in humans and rats (Marin-Kuan *et al*., 2007; 2011).

Cavin *et al*. (2007) reported that a number of genes down-regulated by OTA contain antioxidant regulatory elements (AREs) in their promoter regions which are recognized by the Nrf2-genes. These findings further support that OTA inhibits the Nrf2-mediated gene expression pathway which inturn leads to an impaired antioxidant defense in cells increasing the susceptibility of cells to oxidative damage by free radicals (Guo *et al*., 2014) and may be the reason for the carcinogenic properties of OTA (Cavin *et al*., 2007). In addition, FB_1 exposure has also been reported to trigger the activation of nuclear factor-kappa (NF-κB) and other transduction pathways which are linked to oxidative stress and carcinogenicity (Gopee and Sharma, 2004). The question is whether the inclusion of antioxidants from herbal sources in animal feeds can have any significant protection against mycotoxin-induced oxidative stress and related effects?

The liver and kidney are the two main target organs for mycotoxins; therefore these organs are also expected to express the attributes of the speculated mycotoxin-

induced oxidative stress. This is because mycotoxin metabolism mainly occurs in the liver and the subsequent biotransformation products generated therein are discharged in the kidneys. In addition, a very recent study by Hou *et al*. (2013) on the effects of consumption of multiple mycotoxins (AF, ZEN and DON) naturally contaminated maize in mice suggests the expression of mycotoxin-induced oxidative stress in the kidney and liver. They reported elevated glutathione peroxidase (GPx) activity and malondialdehyde (MDA) level in serum and liver, decreased catalase (CAT) activity in serum, liver and kidney and decreased superoxide dismutase (SOD) activity in the liver and kidney. This thus further confirms the occurrence of mycotoxin-induced oxidative stress expressed on these organs.

2.2.8 Generalized effects of mycotoxins

2.2.8.1 Health effects

In general, mycotoxins are known to cause acute and chronic intoxication in animals and humans (Viljoen, 2008) and animals suffer more because they are fed the poorest quality grades of grains (D'Mello *et al*., 1999). Some of the key effects in poultry are increases in stillbirth and embryonic mortality. The immunosuppressive effects of mycotoxins reported include decreased antibody production against antigens, and impaired delayed hypersensitivity response, reduction in systemic bacterial clearance, increased colonisation of pathogenic microorganisms. All these immunotoxic effects increase the susceptibility of poultry to infectious diseases (Oswald *et al*., 2005; Girish and Smith, 2008).

Mycotoxins also affect the health of humans, when consumed from plant and animal derived products (Yiannikouris and Jouany, 2002; CAST, 2003; Zain, 2011). They may also pose a threat, though to a lesser extent, when dust contaminated with toxins is inhaled (another route of exposure) (Jarvis, 2002). Frequently, they are described as either being toxic, mutagenic, teratogenic, immunosuppressive and or carcinogenic (Eaton and Groopman, 1994; Bhatnagar *et al*., 2002).

Diseases caused by mycotoxins are generally described as mycotoxicoses. The most frequently reported are PN/CN aflatoxicosis and equine leukoencephalomalacia (ELEM). They can be chronically based on intake level, duration of exposure, kind of toxins, animal species, age of the exposed individual, etc. (Hussein and Brasel,

2001). Mycotoxicoses is often associated with high mortality incidents among affected animals (Stoev, 2013). The major contributor to high spontaneous animal mycotoxicoses has been linked to opportunistic secondary bacterial infections that occur as a result of the immunosuppressive action of mycotoxins (Deshmukh *et al*., 2005; Girish and Smith, 2008, Stoev and Denev, 2013).

All of these affect significantly the feed and animal industry, regardless of the scale. This is very typical in the developing countries where food resources are scarce, and the population battles below the breadline enabling the condemnation of contaminated feeds almost impractical (Fapohunda, 2011). Also, these countries tend to export the best grain retaining the poorest quality for domestic consumption (Negedu *et al*., 2011; Stoev, 2013), thus, the problem of mycotoxin exposure can be socio-economically related.

2.2.8.2 Socio-economic effects

The costs of mycotoxin contamination are estimated to be in millions of US dollars in losses worldwide annually (Zain, 2011). In the United State, *Fusarium* contamination in wheat and barley, are approximated to be as high as 2,900 million US Dollars (Negedu *et al*., 2011; Moretti *et al*., 2013). It has also been reported that at least 25-40% of cereals are contaminated with mycotoxins, globally, annually (Pittet, 1998; Yiannikouris and Jouany, 2002; Lawlor and Lynch, 2005). There are various indications that most of these contaminated cereals when condemned are very often used as animal feed components. This results in low productivity thus reducing the profit margins as most African populations rely on subsistence farming, this impacts seriously on their socio-economic wellbeing and livelihood.

Binder *et al*. (2007) reported an alarming level of 52% mycotoxin contaminations in animal feed from Europe and the Mediterranean. This obviously affects the profitability of livestock production. For instance, in North Carolina alone, the losses due to mycotoxin feed contamination to the animal production industry for the year 1992 were US$ 20 millions for poultry, US$ 10 millions for swine (Choudhary and Kumari, 2010). One of the effects that lead to economic losses is feed refusal which ultimately causes reduced weight gains, egg production, low fertility and poor carcass quality (Degirmoncioğlu *et al*., 2005; Bryden, 2012). The impact of mycotoxin

exposure in economic terms is also accounted for by increased cost in health and veterinary care services, decreased livestock productivity, disposal of contaminated food and feeds, and research and management of the mycotoxin problem (Hussein and Brasel, 2001; CAST, 2003; Viljoen, 2008). The economic assessment of decreased production, chronic damage to organs and tissues, increased diseases as a result of immune suppression, and interference with reproductively; is reported to be higher than acute death in mycotoxin-exposed livestock (Abdel-Wahhab and Kholif, 2008). It therefore follows that mitigating the mycotoxin problem is crucial.

2.3 Mycotoxin detoxification/decontamination

2.3.1 Decontamination strategies

There has been an ongoing research on the development and implementation of effective pre- and postharvest decontamination strategies (Jard *et al*., 2011). These mechanisms have been classified into three basic possibilities those which include the prevention of contamination, the decontamination of mycotoxin contaminated food and feed, and the inhibition of absorption of mycotoxins within the digestive tract into the various targeted organs. The most common decontamination processes are categorized according to the following subdivisions: physical separation and inactivation, biological inactivation, chemical inactivation, and decreasing bioavailability (Peraica *et al*., 2002; CAST, 2003; Abdel-Wahhab and Kolif, 2008). These strategies have been revised by CAC/RCP (2003 rev. 2014).

2.3.1.1 Physical separation and inactivation

Physical separation includes the mechanical separation of contaminated grains from the lot either by thermal inactivation, density segregation, solvent extraction or irradiation with gamma rays, microwave, or ultra violet light. All these strategies have capacity limitations (CAST, 2003). Thermal treatments do not completely eliminate OTA as reviewed by Amйzqueta *et al*. (2009). Some of the physical strategies like solvent extraction affect the nutritional component of the feed (Abdel-Wahhab and Kolif, 2008).

2.3.1.2 Biological inactivation

There are some few biological measures that have been taken to combat mycotoxins in the field. For example, non-toxigenic strains of *A. flavus* and *A. parasiticus* have been applied in the soil as a biocontrol and showed reduction in pre-harvest aflatoxin contaminations in peanuts (Jard *et al*., 2011). At the International Institute for Tropical Agriculture (IITA), Ibadan in Nigeria, this programme has been successfully implemented in maize fields to control AF in maize by endemic non-AF producing *A. flavus* strains (Atehnkeng *et al*., 2008; 2014). The same experiments were also carried out in the US by Cotty and Mellon (2006) whereby the same non-toxigenic isolates were applied to limit AF in cottonseed. Biocontrol using non-toxigenic *A. niger* strains were also reported to have shown potential in limiting OTA production (Valero *et al*., 2007). Despite the successes, the probability of recombination with toxigenic strains is often the concern at ecological and sanitary level (Amйzqueta *et al*., 2009). Moreover, the same non-toxigenic isolates may also be toxigenic in producing other mycotoxins, like the case of cyclopiazonic acid (CPA) which can be produced by a non- aflatoxin producer, *A. flavus*. Another concern is that due to environmental influences, this non-producing isolate later develops its potential to produce the same mycotoxin, making it difficult to be managed subsequently.

The addition of microorganisms or enzymes capable of detoxifying some mycotoxins has been reviewed by Amйzqueta *et al*. (2009) and Jard *et al*. (2011). There are also numerous reports on genetically engineered fungal resistant crop cultivars that can be planted (Maupin, *et. al*., 2003; Menkir *et al*., 2008) to reduce mycotoxin development in foods, however, these maize lines are currently not commercially viable. Moreover, the efficacy of biocontrol agents is often dependent on environmental conditions and thus, other measures are undertaken in the management of mycotoxins

2.3.1.3 Chemical inactivation/ chemoprotection

The chemicals used are classified as bases (ammonium and sodium hydroxide), oxidizing agents (hydrogen peroxide and ozone), reducing agents (bisulphites and sugars), chlorinating agents (chlorine), salts and miscellaneous agents (formaldehyde) (Abdel-Wahhab and Kolif, 2008). Aflatoxins degradation by ammoniation has been shown to be quite effective in cottonseed and cottonseed

meal. The treatment of aflatoxicol with sodium bisulfite also inactivates aflatoxins. Ozonization is another technique utilized in maize and cottonseed meals and is limited to decreasing DON, moniliformin (MON), CPA, OTA, patulin, secalonic acid D and zearalenone (ZEA). However, chemical degradation has been shown to cause some alterations in the nutritional quality of feed or food. For example, ammoniation causes a decrease in lysine and sulphur containing amino acids (Scott, 1998). Also, the use of chlorophyll and oltipraz as well as esterified glucomanoses has significant limitation (Abdel-Wahhab and Kolif, 2008). In addition, toxicity of chemical fumigants like ethylene dioxide and methyl bromide used for postharvest preservation purposes, have been reported (Bhat *et al*., 2010). These thus limit their applications against mycotoxins.

2.3.1.4 Decreasing bioavailabitity/enterosorption

Mycotoxin clay binders principally interfere with the structure and bioavailablility of mycotoxins making them unavailable for absorption in the gastrointestinal tract. Scott (1998) provided some properties of binders. Accordingly, during the decontamination process, these feed additives must be capable of destroying, inactivating or removing the mycotoxin, and not produce or leave toxic, carcinogenic or mutagenic residues in the final products or in the food products obtained from animals fed decontaminated feeds. Further to these, they are to retain the nutritive value and acceptability of the product, not substantially alter important technological properties, should destroy fungal spores and mycelia which could under favourable conditions, proliferate and form new toxins. In addition, the processes should be readily available, easily utilized and inexpensive.

Calcium amminosilicates have been reported to effectively bind aflatoxins although a greater concern is the absorption of micronutrients in the process (Abdel-Wahhab and Kolif, 2008). Cholestryramine has been reported to be effective in binding zearalenone, while clay and zeolite minerals are being tested for their effectiveness to target this estrogenic mycotoxin. Activated carbon was reported to have no effect in the treatment of FB_1 contaminated feed (Piva *et al*., 2005). Kolossova *et al*. (2009) also reported no significant absorption of mycotoxins by binders in animal feeds.

Recent breakthrough in terms of the treatment of a broad spectrum of mycotoxins using a single dose treatment has been claimed by mycotoxin binders including Bio-Bantox and Bio-Bantox plus (hydrated sodium and calcium aluminosilicate (HSCAS)). This product works at 5 ppm of toxin per 2.5 kg/Mt is 97% for citrinin, 95% for AF, 94% for T-2, 84% for vomitoxin, 72% for OTA, 70% for FB_1, and 43% for ZEA (Barman, 2014). Mycosorb another binding agent administered at 0.2% has been shown to effectively prevent the intestinal absorption of a mixture of *Aspergillus* and *Fusarium* toxins in broilers and pigs (Smith *et al*., 2006). Despite these advances, the concern remains with the affordability and accessibility of such products to developing countries, especially to subsistence farmers within the sub-Saharan African region.

The most important aspect of these strategies is that the agent has to detoxify mycotoxins by destroying, modifying or absorbing them enough to prevent any toxicity (Huwig *et al*., 2001). These processes are not just straight forward, but driven by enzymes that essentially require cofactors, coenzymes and other molecules (Liska, 1998). In the case of biological control strategy, it is important to consider what the end products of enzymatic breakdown are and what happens if they react with other molecules interfering with vital physiological functions or even initiating further tissue damage.

A new functional group of feed additives was defined by the Commission Regulation of the European Commission (EC) No. 386/2009 as ‘substances for reduction of the contamination of feed by mycotoxins: substances that can suppress or reduce the absorption, promote the excretion of mycotoxins or modify their mode of action (EFSA, 2010a). Depending on their mode of action, these feed additives may act by reducing the bioavailability of the mycotoxins (EFSA, 2010a), degrading them or transforming them into less toxic metabolites (Jard *et al*., 2011).

2.3.2 Herbal protection

The main purpose of herbal protective approach is to safely utilize animal feeds contaminated with mycotoxins in order to reduce farm loses. The proposed herbal additives fall under dietary interventions (Riley and Norred, 1999), based on recent

developments that plant extracts give some cytoprotection to mycotoxin-induced oxidative stress (Periasamy *et al*., 2014). These include but not limited to the significant protective effects of feed additives derived from the aqueous extract of artichoke, sesame seed, roxazyme-G and L-3phenylalanine reported to effectively reduce the growth inhibitory effects of OTA (Stoev *et al*., 2002a). These additives also enhance taste and flavor of feeds and promote digestion (Uniyal *et al*., 2006), therefore improve the overall animal performance, seemingly altered by mycotoxins. A number of plant extracts reviewed by Amйzqueta *et al*. (2009) and Hamzah *et al*. (2013) have shown that natural antioxidants have the potential to reduce the cytotoxic effects associated with exposure to OTA. However, controversy concerning their efficacy exist (Bolt and Stewart, 2012).

2.3.2.1. Herbal plants as sources of drug scaffolds

Various plant phytochemical compounds that play a significant defensive role against herbivory and pathogen attack, inter-plant competition, and abiotic stresses (Kaufman *et al*., 1999; Duke and Bogenschutz-Godwin, 1999) can be utilized for therapeutic purposes (Briskin, 2000). This is because, plant phytochemicals possess enormous scaffolds that are mimicked in the design of most molecular stuctured synthetic drugs (Balunas and Kinghorn, 2005; Mishra and Tiwari, 2011) or even modified further to enhance a drug's biological activity profile (Itokawa *et al*., 2008). Thus, there has been a renewed interest in investigating natural products as leads for new biologically friendly, therapeutic drug candidates (Mishra and Tiwari, 2011).

On a wide basis, at least 25-50% drugs (Upadhyay, 2011) have been extracted from higher plants, or synthetically modified further (Srivastava *et al*., 1996). Natural plant product remedies are popularly preferred as they are widely available, economically viable, and environmentally friendly with fewer side effects (Upadhyay, 2011), although, some of them are now being made synthetically for economic gains (Newman *et al*., 2000). A wide range of medicinal plant parts are targeted for extraction, including leaves, roots, flowers, fruits, twigs and exudates (Uniyal *et al*., 2006). The extraction solubizes secondary metabolites in the form of several of such polyphenols as tannins, phenols, terpenoids, flavornoids, saponins and steroids that have complex structures involving many chiral centers that determine their biological

activity (Bruneton, 1995). The structure of flavanoids for example, has been linked to antioxidant activity of herbal extracts.

Flavonoids are a large group of phenolics that occur naturally in plants, characterized by the carbon skeleton C6-C3-C6 with the ability to modulate various enzyme activities (Gülçin, 2012). Generally, the ability of flavonoids to effectively act as antioxidants depends on a number of factors, i.e., metal-chelating potential that strongly depends on hydroxyls and carbonyl groups arrangement around the molecule, the hydrogen or electron-donating substituents present and able to reduce free radicals, and the flavonoid's ability to delocalize unpaired electron which lead to stable phenoxyl radical formation (Seelinger *et al*., 2008; Gülçin, 2012).

Mohammed *et al*. (2013) stated that the total phenolic content in the plant extract is linked to antioxidant capacity, which is also a function of the structures of phenolic compounds. Basically, phenolic antioxidants (PPH) inhibit lipid peroxidation through a rapid donation of the hydrogen atom to the peroxyl radical (ROO). This results in the formation of alkyl (aryl) hydroperoxide (ROOH) as illustrated in the following reaction: ROO + PPH → ROOH + PP (Wang *et al*. 2007). In addition, the steric and electronic forces within the structure of for example, phenols have been reported to be responsible for the antioxidant activities exhibited by herbal plants (Barclay *et al*., 1993).

2.3.2.2 Herbal plants as sources of antioxidants

In recent years, there has been a growing interest in identifying alternative natural and safe sources of diet antioxidants, especially from herbal plant origin (Santos *et al*., 2009; Verma *et al*., 2010; Gülçin, 2012; Parimala and Shoba, 2013; Uddin *et al*., 2014). An antioxidant is a molecule capable of inhibiting the oxidation of other molecules (Flora, 2009). Dietary antioxidants have been defined as any substance that when present in low concentrations than that of the oxidizable substrate, significantly delays or inhibits the oxidation of that substrate (Halliwell and Gutteridge 1995; Institute of Medicine (US) Food and Nutrition Board, 2000; Halliwell, 2007). They ultimately stimulate cellular defenses protecting cellular components against oxidative damage (Halliwell and Evans, 2001; Dudonnè *et al*., 2009).

The consumption of plant-sourced antioxidants has been linked to decreased incidence of degenerative diseases such as cancer, coronary atherosclerosis and Alzheimer's disease (Wang *et al*., 2007; Nikolova, 2012; Uddin *et al*., 2014). Vitamin C, carotenoids, and polyphenolic substances (anthocyanins, flavonols) play a crucial role as they possess radical scavenging activities and are thus able of neutralizing harmful effects of lipid peroxidation or DNA damage caused by free radicals (Terry, 2001; Santacruz *et al*., 2012; Uddin *et al*., 2014).

All aerobic organisms have antioxidant defenses, including antioxidant enzymes and antioxidant constituents that are activated in the presence of oxidative stress (Davies, 1995). Some of these antioxidant enzymes have been evaluated as new types of dietary natural antioxidants (Nimse and Pal, 2015), which can be applied to manage mycotoxins and associated problems. For instance, caffeic acid was shown to reduce 95% of aflatoxin production by *Aspergillus flavus* (Kim *et al*., 2008).

2.3.2.3 Anti-cancer, anti-inflammatory, and hepatoprotection

Vitis vinifera leaf extracts have also been shown to have protective effects against OTA-induced hepatoma and reneal carcinoma in mice (Jeswal, 1998). Seelinger *et al*. (2008) displayed specific anti-inflammatory and anti-carcinogenic effects of luteolin, explained by its antioxidant capacities. The carcinogenic, inflammatory and cytotoxicity effects due to mycotoxin exposures are a major concern, thus research on herbal active components (phytochemicals), inclusive of flavonoids, with anticarcinogenic, anti-inflammatory and hepatoprotective effects is valuable. However, more investigations on the subject are required to clarify some of the key concepts of flavonoid activities in this respect. In addition, Surh *et al.* (2005) suggested that many phytochemicals enhance cellular antioxidant capacity through the activation of Nrf2 as discussed in Section 2.8.1.4, thereby blocking initiation of molecular links between inflammation and cancer.

Chemicals that cause liver injury are called hepatotoxins (Gargoum *et al*., 2013) and hepatoxicity is one of the predominant effects of mycotoxin exposure, particularly in the case of OTA and AFB_1 (Gupta *et al*, 2008; Khan, 2010; Thakur, 2010). This occurs mainly through mycotoxin-induced lipid peroxidation which ultimately results in damaged membranes of liver cells and necrosis of hepatocytes (Atroshi *et al*.,

2000). The hepatoprotective activity is usually coupled with antioxidant activity. For example, sea buckthorn (*Hippophae rhamnoides*) oil extract was reported to diminish the adverse effects of exposure to AFB_1 in broilers (liver damage) both by antioxidant and hepatoprotective activity (Solcan *et al*., 2013).

2.3.2.4 Antifungal and antimycotoxin agents

The increasing incidence of mycotoxins and mycotoxicoses outbreaks has given a fresh impetus to research targeted at novel antifungal agents. This essentially calls for the development of agents that will either protect crop infestations by fungal pathogens or reduce mycotoxin production thereafter. In more general terms, whether it requires direct control by preventing the occurrence and action of fungi in crops or indirectly detoxifying mycotoxins in contaminated feeds and foods.

Antifungal activity is said to be derived from the inactivation of sulfhydryl in amino acids, which inhibits protein synthesis and important enzymes in the fungal pathogen. The concept of existing antifungal compounds isolated from grape plants e.g. caffeic acid, chlorogenic acid, pterostilbene, resveratrol, and viniferin is that they evolved as defenses against fungal invaders that plaque grape plant (Duke and Bogenschutz-Godwin, 1999).

African plants have been identified as good candidates for the search of active antifungal components, since they survive in all manner of hostile environments. It has been noted that their roots usually have good activity compared to the stem or the leave extracts because, it is directly in contact with the soil. The various studies conducted on antifungal properties on some African plants have revealed the following types of constituents as having significant antifungal activity monoterpenes, diterpenes, quinonoids, triperpene glycosides (saponnis), flavonoids and chalocones, tannins (Hostettmann *et al*., 2000).

Choudhary and Kumari (2010) reviewed on the progress made on plant-based research involving antifungal and antimycotoxin agents. This includes aqueous plant extracts from cinnamon, pippermint, basil, origanum, epizote, clove, and thyme. Caffeine appears to inhibit AF synthesis by restricting the uptake of carbohydrate, which are ultimately used by the mould to synthesize AF. Capsanthin isolated from

Capsicum annum (red chilli) was also reported to inhibit fungal growth and mycotoxin production. Phenolics like tannic acid, caffeic acid and phloroglucinol at 0.01 M concentration prevented AF production by more than 55%. One of the compounds from cinnamon, *O. methoxycynamaldehyde* has been reported to be highly effective against *A. flavus* and *A. parasiticus* as reviewed by Dwivedi and Singh (2011).

Bilgrami *et al*. (1992) reported the inhibition of mycelial growth and AF production on maize grains when treated with onion, garlic and eugenol. Bankole and Joda (2004) observed that *Cymbopogon citrates* powdered leave extracts and the essential oil fraction both had significant antifungal activity against *A. flavus* and also had AF production inhibited. Recently, components of *Eucalyptus* oil limonene and geranial were confirmed to have antifungal potential *as* reviewed by Choudhary and Kumari (2010). The leave extract of *Allium sativum* L. x *Allium cepa* L. also exhibited antifungal and antimycotoxin (AFB_1) activity against *A. flavus* (Sandosskumar *et al*., 2007). The essential oil extracts of *Ageratum conyzoides* also exhibited antifungal and antimycotoxin activity against *A. flavus and A. parasiticus* (Adjou *et al*., 2012). Murugan *et al*. (2013) reported the antiaflatoxigenic food additive potential of the methanol extract of *Murraya koenigii* which displayed antifungal activity and inhibited AFB_1 production by 99.6%.

2.3.2.5 Herbal extracts in the treatment of other toxins

Herbal extracts are also effective in reducing the effects of oxidative stress induced by not just mycotoxins but other toxic components as well. For instance, Yu *et al*. (2011) reported significant hepatoprotective activity of flavanone, chalcone, and triterpene acid named 2′, 4′-dihydroxy-6′-methoxy-3′, 5′-dimethylchalcone (DMC) extracted from the buds of *Cleistocalyx operculatus* (Roxb.). In that study, the increased levels of MDA, ROS, and PCC in the liver of CCl4-treated experimental rats were lowered by treatment with DCM. This was attributed to the hydroxyl groups present in DMC. The hepatoprotective effects of DMC were related to the attenuation of oxidative stress, by increasing the rate of the antioxidant cascade and inhibiting lipid peroxidation in the liver. The same principles are essentially proposed to apply to the reduction of mycotoxin-induced oxidative stress effects using natural antioxidants sourced from herbal plant components. The following herbal plants are

reviewed as potential sources of natural antioxidants, and focus of this study, for the development of herbal formulations for reducing animal exposure to mycotoxins and their health effects thereafter.

2.3.3 *Mentha longifolia* (Labiatea)

Mentha longifolia is widely used in most parts of the world as a domestic herbal remedy (Ali *et al*., 2006) particularly in southern African traditional medicine (van Wyk *et al*., 2009). It has been suggested that the phytochemical constituents of this plant have beneficial effects which may act as growth promoters, inhibit intestinal pathogenic organisms, and improve digestion and absorption in broilers (Al-Ankari *et al*., 2004). The leaves and flowers of this plant are rich in phenolic natural antioxidants (Tekeľová *et al*., 2009). Thus, *M. longifolia* potentially has active components that can be of value in the development of feed and food additives (Hajlaoui *et al*., 2009) to reduce the effects of mycotoxin exposure in animals.

2.3.3.1 Botanical description and distribution

Mentha longifolia is described as perennial herbs that has creeping rhizomes, and erect flowering stems. It can reach up to 1.5 m in height under optimum growth conditions but normally grows in the range of 0.5-1 m in height (van Wyk *et al*., 2009; Al-Ankari *et al*., 2004). All parts are highly aromatic with a strong typical mint ordor (van Wyk *et al*., 2009) and a pungent with slightly bitter taste (Al-Ankari *et al*., 2004). Leaves appear opposite to each other in pairs along the stems, which are squares in cross sections. The soft leaves are described as coarsely hairy, ovate and lanceolate and the edges sparsely toothed between 45-100 mm long, and 72 mm wide. The colour of the leaves varies from light dark green to grey. The small white or pale purple flowers are borne in elongated clusters at the tips of the stems (van Wyk *et al*., 2009; Ashfaq *et al*., 2012; Voon *et al*., 2012).

Mentha longifolia is widely distributed in South Africa (van Wyk *et al*., 2009). It is also common in other parts of southern Africa, Europe, the Mediterranean region and eastern parts into Asia (Al-Ankari *et al*., 2004; Razavi *et al*., 2012) as well as in temperate parts of Eurasia and Australia (Ali *et al*., 2006). *Mentha longifolia* is known as ballerja, kruisement (Afrikaans), wild mint, biblical mint, horsemint (English) (Duke

et al., 2002), koena-ya-thaba (Sotho), inixina (Xhosa), ufuthana lomhlange (Zulu) (van Wyk *et al*., 2009). It is commonly known as 'puneh' in Iran (Razavi *et al*., 2012) and Konjski bosiljak, or Dugolisna nana in the Balkan region (Šarić-Kundalić, 2010).

2.3.3.2 Phytochemistry

The phytochemical screening of *M. longifolia* extracts revealed the following bioactive chemical constituents alkaloids, flavonoids, cardiac glycosides, phenolics, tannins, monoterpene ketones, saponins and terpenes (Ghoulami *et al*., 2001; DoNascimento *et al*., 2009; Ashfaq *et al*., 2012). The major phenolic constituents are caffeic acid derivatives (Tekeľova *et al*., 2009) and polar flavonoids (Ghoulami *et al*., 2001; Akroum *et al*., 2009; Tekeľova *et al*., 2009).

The flavonoids described within *M. longifolia* are flavones, flavanones and their glycosidic forms including luteolin, apigenin, eriodictyol, hesperetin and their glycosides (Tekeľova *et al*., 2009) as well as quercetin and kaempferol glycosylated derivatives (Al-Bayati, 2009). These compounds are attributed to the plant's antimicrobial activities. It has been shown that different glycosylated flavonoids exert a synergistic effect on antimicrobial activity (Al-Bayati, 2009; Razavi *et al*., 2012).

The presence of polyphenols, flavonoids and condensed tannins in this plant is correlated with the high antioxidant activities in its aerial part (Hajlaoui *et al*., 2009), although van Wyk *et al*. (2009) reported a rather weak antioxidant activity. High levels of hydroxycinnamic derivatives (THD) linked to antioxidant scavenging capacity (Gaspar *et al*., 2009) were observed in *M. longifolia* (Tekeľova *et al*., 2009). Also, *M. longifolia* contains bioflavonoids such as hesperidin said to improve capillary function by relieving capillary impairment and venous insufficiency of the lower limbs (Petkar and Viljoen, 2008). Ali *et al*. (2006) reported ceramides isolated from this plant.

The volatile oils of *M. longifolia* contain monoterpeniods such as carvone, limonene, methone and menthol (van Wyk *et al*., 2009) as well as octanol, linalool, terpinene, piperitone (Šarić-Kundalić, 2010). Volatile oils are known for their decongestant and antiseptic effects though they will not be discussed in this review.

2.3.3.3 Pharmacology

Phenolic compounds of mints poses a wide array of pharmacological properties, i.e., they may perform one or more of antioxidant (Nickavar *et al*., 2008; Mimica-Dukic and Bozin, 2008; Vladimir-Knežević *et al*., 2014; Ertaş *et al*., 2015), antiulcer, cytoprotective, cholagogue, chemopreventive, anti-inflammatory, antidiabetogenic (Mimica-Dukić and Bozin, 2008), antimicrobial (Al-Bayati, 2009) and heptoprotective (Mimica-Dukić *et al*., 1999; Mimica-Dukić and Bozin, 2008) activities. These attributes are desirable for use of this plant as an agent against the effects that could arise from mycotoxin exposure in humans and animals.

Mabona *et al*. (2013) reported antimicrobial activity of *M. longifolia* against *Staphylococus aureus, Streptococcus epidermidis, Pseudomonas aeruginosa, Candida albicans, Brevibacillus agri, Propionibacterium acnes*, *Trichophyton mentagrophytes*, and *Microsporum canis.* It was interesting to note that *M. longifolia* also possess excellent inhibitory effects against plant pathogen *E. carotavora and S. sclerotiorum. Erwinia carotovora* is a problematic bacterium that causes soft rot in fruits and vegetables (Strange, 2003), while *S. sclerotiorum* is a prevalent pathogenic fungi causing plant stem rot as reviewed by Razavi *et al*. (2012). Thus, *M. longifolia* is considered a naturally derived potential biopesticide candidate, especially since the use of synthetic pesticides has negative effect amongst others being the development of resistance (Razavi and Nejad-Ebrahimi, 2009).

2.3.3.4 Medicinal applications

Mentha longifolia is known as a traditional remedy for coughs, colds, asthma and other respiratory ailments. It is also used as therapy against fever, indigestion, flatulence, urinary tract infections, painful menstruation (Voon *et al*., 2012), headaches, hysteria and birth stimulant in delayed pregnancies, (van Wyk *et al*., 2009; Philander, 2011). It is believed to be diaphoretic and antispasmodic and externally used for treating wounds and swollen glands (van Wyk *et al*., 2009; Philander, 2011). In Iran folk medicine for centuries, *M. longifolia* has been described as a tonic, carminative, digestive, stomachic, antispasmodic and an anti-inflammatory agent (Razavi *et al*., 2012). The teas or decoctions prepared from the plant material have been shown to increase diuresis and flatulence, treat metabolism, liver and gall disorders, musculosceletal system disorders and rheumatism (Šarić-Kundalić, 2010)

amongst other effects already discussed. All these medicinal uses collectively indicate some potential active scaffolds in this plant that can be investigated further for inclusion in animal feeds in order to reduce animal exposure to mycotoxins and their adverse effects thereof.

2.3.4 *Leonotis leonurus* (L.) R. Br. (Lamiaceae).

Leonotis leonurus has been traditionally use in the Eastern Cape as a vertinary remedy. According to van Wyk (2013), it is added to drinking water to prevent sickness in domestic animals (Hirst and Knott, 2007). The infusions of leaves, sometimes mixed with leaves of *Cutia hirsute* E. Meu.Ex-Sond, were shown to be effective in the treatment of gall sickness in cattle (Hirst and Knott, 2007; van Wyk *et al*., 2009). The leaves and flowers are also effective in the treatment of tapeworms (Hirst and Knott, 2007) and eye inflammation (Masika *et al*., 2000).

2.3.4.1 Botanical description and distribution

Leonotis leonurus is widely known as 'lion's tail', 'lion's ear' or 'wild dagga' which was coined from the Latin name *leonurus,* meaning lion's ears as the flowers resemble lion's ears (Hutchings *et al*., 1996; van Wyk *et al*., 2009). This plant has been described by Ivarsson (1985) and van Wyk *et al*. (2009) as a shrub that grows to between 2-5 m in height, branching from a thick woody base with a pale brown and densely pubescent stem. Its leaves are opposite, petiolate, coriaceous, linear, acute at apex and base and serrate in the distal half. The upper surface of the leaf is bright green and the lower part densely pubescent. Bright orange, tubular flowers are borne in characteristic rounded whorls on spiky bracts of erect stems. It is native to southern Africa, especially in the Eastern Cape, Western Cape, Kwazulu-Natal, Mpumalanga, and Gauteng. *Leonotis leonurus* is also commonly known as cape hemp, wild dagga (hemp) (Duke *et al*., 2002), minaret flower, koppieddagga/ rooidagga (Afrikaans), umfincane (Xhosa), imunyane (Zulu) and lebake (Sotho) (Hutchings *et al*., 1996; Hirst and Knott, 2007; van Wyk *et al*., 2009).

2.3.4.2 Phytochemistry

The main phytochemical compounds isolated from *L. leonurus* include tannins, quinones, saponins, alkaloids, terpenoids, triterpene and steroids as reviewed by Ascensao and Pais (1998) and Wu *et al*. (2013). The plant also contains about 0.15-0.18% volatile oils, which are primarily responsible for its peculiar scent (Hirst and Knott, 2007). A number of studies have demonstrated the isolation of labdane diterpenoids from its leaves. For example, He *et al*. (2012) uncovered 3 new labdane diterpenes from commercial aqueous extract, associated with anticonvulsant effects suggested by observed gamma-aminobutyric acid ($GABA_A$) activity. Wu *et al*. (2013) reported 8 new labdane diterpenoids which could be responsible for its psychoactive properties.

The most reviewed diterpenoids in *L. leonurus* are the furanic labdane type lactones, premarrubiin and marrubiin (Hirst and Knott, 2007). Generally, the anti-inflammatory activity of diterpenoids is caused by their ability to suppress nuclear factor-κB (NF-κB) signalling, which is the major regulator in the pathogenesis of inflammatory diseases (Salminen *et al*., 2008), including those associated with cancer and mycotoxin exposure. The diterpene marrubiin isolated from a South African *L. leonurus* was reported to possess anti-inflammatory properties, through the suppression of NF-κB signalling pathway (Mnonopia *et al*., 2011).

Oyedemi and Afolayan (2011) reported that the high phenolics, flavonoids, proacyanidins and flavonols content showed a positive linear correlation with antioxidant activity of *L. leonurus* extracts. This supports the view that it can be used to prevent mycotoxin-induced oxidative stress-related effects. Oyedemi *et al*. (2011) reported the presence of high phytochemicals in this plant especially phenolics and flavonoids, those that can be associated with antidiabetic activity. This was shown with the observed reduced blood glucose and lipids levels, improved polydipsia, polyuria in diabetic rats. These finding agree with the data signifying that *L. leonurus* alleviates diabetic symptoms (Mnonopia *et al*., 2012).

2.3.4.3 Pharmacology

The pharmacological mechanisms of action of *L. leonurus* have not been fully clarified (Martindale, n.d). Premarrubiin and marrubiin have been reported to be

responsible for increased perspiration, secretion of saliva and gastric juices. Interestingly, these aspects are similar to those described as the bitter tonic effect. Additionally, due to their antibacterial, antifungal, antioxidant, immunological influences and hepatoprotective properties, a number of plants have also been attributed to exhibit bitter tonic effects (Olivier and van Wyk, 2013). Some of these properties are desirable in formulating feed additives derived from herbal plants.

The effects of *L. leonurus* aqueous extract on blood pressure and heart rate were investigated by Mugabo *et al*. (2012), who confirmed the presence of constituents in the extract that are associated with a positive inotropic, negative chronotropic and coronary vasolidation, even when administered at low concentrations. Other findings on *L. leonurus* provide evidence of its anticonvulsant, antioxidant, antibacterial, hypoglycaemic and cardioprotective effects as reviewed by Mnonopia *et al*. (2011). The aqueous extracts of dried leaves of *L. leonurus* at dosage of 200 mg/kg, have demonstrated significant anticonvulsant activity in mice (Bienvenu *et al*., 2002). Marrubenol and marrubiin have been implicated to be potent *in vitro* in the inhibition of KCl-induced contraction of rat aorta as reviewed by Hirst and Knott (2007).

2.3.4.4 Medicinal applications

Leonotis leonurus has a variety of traditional medicinal uses including the treatment of coughs, colds, influenza, chest infections, diabetes, hypertension, eczema and other skin diseases, epilepsy, delayed menstruation, intestinal worms, constipation, spider bites, scorpion stings, sores, boils and snake bites (Hutchings *et al*., 1996; van Wyk *et al*., 1997, 2009; Afolayan and Sunmonu, 2010). The treatment of asthma, bronchitis, high blood pressure, headaches, viral hepatitis and cramps using this plant's parts has also been reported (van Wyk, 2008). An interesting group of herbalists found in the botanically diverse parts of the Western Cape of South Africa claim that *L. leonurus* treats cancer, ulcers, aches, pains, gout, and dandruffs and also it is taken as a blood purifier (Philander, 2011). The leaves and flowers were chewed to relieve epigastric pain (van Wyk and Grecke, 2000). *Leonotis leonurus* has also been reported to be effective in the treatment of gastrointestinal hemilnthosis (Maphosa *et al*., 2010).

2.3.5 *Piptedaniastrium africanum* (Hook.f.) Brenan

This plant resembles those of the genus *Piptadenia* with slight differences, thus the word astrium. Interest in the study on *P. africanum* is based on the reported antifungal activity of methanolic and aqueous extracts against *Pyricularia grisea* (rice blast-causing fungi), most of which is ascribed to the presence of an isolated saponin similar to a commercial fungicide, Flutriafol (Brusotti *et al*., 2013). This thus, supports that *P. africanum* has potential in the development of antifungal agents that can be applied against mycotoxigenic fungi.

2.3.5.1 Botanical description and distribution

The botanical description of this plant is provided by Asamoah (2009). Accordingly, *P. africanum* trees can grow up to 50 m in height, characterized with a buttressed base and a smooth grey bark, short branchletls that are densely rusty and pubescent, then glabrescent. The pinnae has 10-19 pairs comprising the leaflets with 30-58 pairs that are linear, falcate, 3-8.5 mm long and 0.8-1.25 mm wide. The flowers are yellowish-white, in spiciform racemes of 4-11 cm long, with flat, thin pods of 17-36 cm long and 2-3.2 cm wide. The pot splits to release flat winged, wind dispersed seeds 3.5-3.95 cm long and 1.8-2.5 cm wide. Its heartwood is light to golden brown and the sapwood is greyish to pale straw. This plant is widely distributed throughout the tropical rainforest of West African countries including Senegal, Sudan, Uganda, Angola, DRC, Nigeria and Cameroon (Fongod *et al*., 2013). *Piptedaniastrium africanum* is known as *Okibomi kachikabiam* in Nigerian languages (Ita and Offiong, 2013) and as small leaf *Dabema* in English (Fongod *et al*., 2013).

2.3.5.2 Phytochemistry

Phytochemical studies on extracts of *P. africanum* have showed the presence of tannins, flavones, alkaloids, steroids, terpenoids, saponosides and glycosides (Mengome *et al.,* 2009; Akinlami *et al*., 2012) as well as carbohydrates, sterols, glycosides (Akinlami *et al*., 2012) and triterpene glycosides (Noté *et al*., 2013). Mengome *et al*. (2009) isolated sapanosides from ethanolic extracts which were reported to inhibit by 50% the growth of human colonic cancer cell line CaCo-2 *in*

vitro. Brusotti *et al*. (2013) isolated saponin fraction with antifungal activity against 5 strains of rice blast fungus *Pyricularia grisea* and the tannin fraction was equally seen to be active against *Staphylococcus aureus* and *Streptococcus mutans*. The expressed radical scavenging activity of this plant extract was due to the presence of constituents such as tannins and flavonoids (Grigorov *et al*., 2014). The hexane and ethyl acetate fractions were reported to have anti-helmitic, antibacterial and antifungal activities (Fred-Jaiyesimi *et al*., n.d).

2.3.5.3 Medicinal application

Interest in the use of this plant in the study is based on its medicinal properties. Mainly, it is used to treat coughs, headaches, mental disorders, stomach aches, male impotence, and to expel worms. *Piptedaniastrium africanum* has also been reported to be used in the treatment of poisoning, tooth aces and as an anti-charm (Ita and Offiong, 2013). It is also used in the treatment of jaundice (Betti and Lejoly, 2009). Betti (2002) described the plant as one of the relative medicinal plant sold at a total cost of 2220 FCFA/kg in Yaoundè markets of Cameroon. Basically, it is for the treatment of anemia, wounds, convulsion, lumbago, meningitis, purgative, and female infertility on a performance index (Ip), 1 denoting the average performance in all these diseases.

2.4 Concluding remarks from the literature reviewed

The reviewed advances made in research that define the possible significance of herbal plant components possessing antioxidant, anti-inflammatory, antifungal, anticancer and hepatoprotective activities in alleviating the plausible mycotoxin-induced oxidative stress and its effects in improving mycotoxin-exposed animals. However, there are still a number of factors and variables that need to be studied further in clarifying the modes of action and activity of phytochemical-containing plants in this regard. The concept of phenolic compounds or phytochemicals with high antioxidant activity as possible additives is still emerging and their use is currently restricted because of solubility or toxicity concerns. In addition, there are still a lot of unanswered questions regarding the mechanism underlying their protective actions (Gaspar *et al*., 2009), especially as feed additives. Furthermore,

the validity of these phytochemical constituents as candidates for the development of antifungal and antimycotoxin scaffolds for inclusion in animal feeds raises numerous unanswered questions at this stage, which definitely require further investigations.

CHAPTER THREE

3. MATERIALS AND METHODOLOGY

3.1 Introduction

The chapter describes the experimental and analytical techniques used in the study. These include plant material collection, sequential solvent extraction, phytochemical analysis, biological assays, antioxidant, and cytotoxicity studies. Experimental techniques used to characterize phytochemicals present in each plant sample include thin layer chromatography (TLC), ultraviolet visible spectroscopy (UV-Vis), fourier transform-infrared absorption spectroscopy (FT-IR), and 2 dimensional time of flight mass spectroscopy gas chromatography (2D GCxGC-TOF/MS). The antioxidant activity of the plant extracts using electron paramagnetic resonance spectroscopy (EPR) is also described. The EPR studies (both *in vitro* and *ex vivo*) were conducted at the Medical Faculty laboratories, Trakia University in Bulgaria, under the International Research Staff Exchange Scheme (IRSES) Call: FP7-PEOPLE-2012-IRSES project. The cytotoxicity studies were conducted using MTT assay to establish the possible *in vitro* effects of herbal plant components as well as to evaluate the efficacy of the hypothesized herbal protection against the effects of mycotoxins OTA and FB_1 on human lymphocyte cells.

3.2 Materials

The solvents used for extraction were of standard grade and were purchased from Merck, South Africa. These included: hexane, dichloromethane, ethyl acetate and methanol. The plant materials extracted were powdered leave extract of *Mentha longifolia*, *Leonotis leonurus* and stem bark of *Piptadeniastrium africanum.* Potato dextrose agar (PDA) purchased from Merck, South Africa was used to inoculate the following *Aspergillus* species, i.e., *A. flavus*, *A. fumigatus, A. ochraceus*, *A. parasiticus* and *A. niger* for the biological assays conducted in the study. Indicator sprays used on the 60 F_{254} TLC and DC-Fertigplatten SIL.G-200UV_{254} 20 x 20 cm Macherey-Nagel plates were vanillin sulphuric acid and p-iodonitrotetrazolium violet (INT).

The equipment used for analysis included rotor evaporator, lamina airflow hood, incubator, centrifuge, UV/Vis spectroscopy, FT-IR spectroscopy, 2D GCxGC-TOF/MS. These analytical instruments were provided by the Department of Applied Chemistry for chemically related works and for biological analysis, by the Food and Health Research Group Laboratories of the Faculty of Health Sciences of the University of Johannesburg. The X-band EMX^{micro} spectrometer Bruker, Germany, equipped with a standard Resonator, Bruker WIN-EPR and Simfonia software, and Transilluminator-4000 equipment (Stratagene, USA) used for antioxidant activity studies was provided by the Medical Faculty laboratories, Trakia University in Bulgaria.

3.3 Methodology

3.3.1 Plant collection

A permit to collect and convey protected and endangered plants (CITES) for scientific purposes was issued in terms of the provisions of the Nature Conservation Ordinance 12 of 1983, by the Premier of the Province of Gauteng Nature Conservation (CPF3 No. 0203). This permit was used to gain access to the South African plant species *Mentha longifolia* and *Leonotis leonurus* used in the study from the Pretoria Botanical Gardens. The leaves were harvested and dried in the shade away from direct sunlight for a period of 3 months. The dried leaves were then ground into powder using an analyette 3 Spartan pulverisette 0 (FRITSCH), and stored in zipper plastic bags in the shaded dry place to avoid photo oxidation. The ground stem bark of *Piptadeniastrium africanum* from Cameroon was provided by Dr. Ndinteh D. of the Applied Chemistry Department, University of Johannesburg.

3.3.2 Sequential solvent extraction

The ground material of each plant was weighed to approximately 10 g into a 250 ml conical flask containing 100 ml of solvent as recommended (Thring *et al*., 2007; Obeidat *et al*., 2012). Sequential exhaustive extraction with each solvent was conducted, starting with hexane, the least polar and ending with methanol the most polar solvent (Kotze and Ellof, 2002; Tiwari *et al*., 2011; Mpofu *et al*., 2014). The procedure involved soaking each plant material in solvent for up to 7 days with mild

daily shaking, thus increasing the exposure and contact of sample surface area with the solvent system (Sasidharan *et al*., 2011). The extract was then filtered through Whatman no. 4 filter papers and the filtrate dried under vacuum using a rotary evaporator (rotavapor R 210, BUCHI). This was then transferred into pre-weighed amber bottles. The extraction yields of each extraction were calculated according to the equation below (Kim *et al*., 2013).

$$\text{Extract recovery (\%)} = \frac{\text{Extract + vial (g)} - \text{Empty vial (g)}}{\text{Amount of plant material used (g)}} \times 100$$

3.3.3 Phytochemical analyses

3.3.3.1 Thin layer chromatography (TLC)

Thin layer chromatography remains popular as the cheapest technique for screening, fractionation and determination of constituents from natural plant products (Hostettmann, 1999). It analyzes mixtures by separating the compounds within the mixture. It is widely used to establish the identity and purity of compounds (Sasidharan *et al*., 2011). The three main steps followed when performing TLC procedures are spotting, development and visualization as described by Kumar *et al*. (2013). Firstly, the sample to be analyzed is dissolved in a volatile solvent to produce a very dilute solution. Spotting, the second step consists of using a micropipette to load a small amount of sample on one end of the TLC plate. Lastly, the development step consists of placing the bottom of an upright TLC plate into a shallow pool of a development solvent, which enables the movement of the fractions up the plate by capillary action. As the solvent moves up, it interacts with the spotted sample and the silica gel. The results depend on the balance between three polarities, i.e., that of the plate, the development solvent and that of the sample. Sample separation is based on the differences in the polarities of the different compounds present and show up as different spots. When the solvent has travelled almost to the top of the plate, the plate is removed, solvent allowed to evaporate and spots marked with a pencil.

The following solvent development systems were used as they were consistent with the fraction's polarity in the study (Eloff *et al*., 2005).

Table 3.1: Solvent development systems

Solvents polarity	Solvent ratio
polar	Ethyl acetate: methanol: water (EMW) (10:1.35:1)
Intermediate polarity	Chloroform: ethyl acetate: formic acid (CEF) (10:8:2)
Non-polar	Benzene: ethanol: ammonium hydroxide (BEA) (18:2:02)

Visualization of the developed TLC chromatogram was conducted in three steps. First the coloured compounds were directly observed after development. Secondly, invisible bands were viewed under ultraviolet light (UV), since the silica gel on the TLC plate is impregnated with fluorescent material that fluoresces under UV light (254 and 365 nm). The compound spot interferes with the fluorescence and appear as a dark spot on a glowing background. While under UV, the spots were outlined with a pencil. Thirdly, the developed chromatograms were sprayed with vanillin sulphuric acid, heated for 5 min at 110°C (Mpofu *et al*., 2014) for chemical visualization.

The R_f value quantifies the movement of the materials along the plate and it is the distance travelled by the sample spot divided by the solvent migration distance (value lies between 0 and 1). Typically, an effective solvent is one that gives R_f values in the range of 0.3 - 0.7.

3.3.3.2 Ultra violet spectroscopy (UV-Vis)

The maximum absorbance of each plant extract was measured on a Shamadzu UV 2450 spectrometer. Accordingly, the powered samples were reconstituted in an appropriate solvent (methanol) (Sandosh *et al*., 2013). Each was put into a cuvette and absorbance measured at a wavelength range of 200-800 nm.

3.3.3.3 Fourier transform-infrared absorption spectroscopy (FT-IR)

The FT-IR spectrometer detects the absorption bands with characteristic peak values of functional groups present in a sample (Coates, 2000). According to the method

described in Johnson *et al*. (2012), the samples of the 3 plant extracts were assayed in their powder form using a Perkin Elmer spectrum 100 FT-IR spectrometer. A small portion of sample was placed on the germanium piece (sample window) using a spatula. The first single beam spectrum contained absorption bands from both the sample and the background. Thus, the ratio between the single-beam sample spectrum and the single beam background spectrum gives the spectrum of the sample. The sample spectrum was then assigned with absorption frequency bands to the rightful modes of vibrations. Functional groups were identified from an analysis of the absorption bands in the infrared spectrum.

3.3.3.4 2D GCxGC-TOF/MS

Each extract was weighed, reconstituted in HPLC grade solvent and filtered through cotton glass pipette. The filtrate was then placed into a capped GCxGC vials (Merck) and analyzed immediately. An Agilent 7683 ALS auto sampler equipped with 10 ul syringe was used for the injections with an injection volume of 1 ul per sample and three sample washes from 2 different solvents (chloroform and methanol) with no viscosity delay. The instrument used for the analysis was an Aligent Technologies 7890A GC equipped with a LECO cryo-modulator coupled to a Pegasus 4D TOF/MS. The settings used for the 2D GCxGC-TOF/MS are presented in Table 3.2 below.

Table 3.2: The GCxGC-TOF/MS setting specifications

Specification	Setting
Column	30 m x 0.25 mm x 0.25 µm Rxi-5Sil Stabilwax
Injection vol.	1 µl (2 injection per sample)
Carrier gas	Helium 1 cm^3/min for entire run
Oven program	80°C for 1 min increased to 250°C at 15°C/min, hot pulse time 0.80 sec
Modulation period	4 sec
Split	10:1

The samples analysed were the organic phase solvent extracts of the three plants. The sample underwent a dual phase separation (GC columns) with two orthogonal phases separated by a cryo modulator and then the various components of the plants were detected by a Time of Flight Mass spectrometer, generating individual mass spectra which were then compared to some databases (NIST, Adams EO library).

3.3.4 Biological assays

3.3.4.1 Media preparation

Potato dextrose agar (PDA) was prepared and sterilized at 121°C for 15 min at 15 Pascal. After cooling, approximately 20-24 ml was aseptically poured into petri dishes in the laminar air flow hood, and stored overnight in a refrigerator.

3.3.4.2 Fungal inoculum quantification

The fungal inoculum was prepared by inoculating a swab full of fungal spores of each of the 5 *Aspergillus* species into separate tubes with ringer solution. The fungal spores were quantified on a hemocytometer slide (BOECO 1/10 mm). A new glass cover was placed over the counting area of the slide. A 20 µl of fungal inoculum was pipetted into the slide by placing the pipette tip at the edge of the slide and allowed to spread into the counting area by capillary action. This was then placed under a microscope (Zeiss) at 100x magnification and focused to count the spores within the 16 square areas. The inoculum was then quantified and adjusted to at least 100-300 spores using the following equation:

C = n/v where C= concentration (spores/ml) n= number of cells counted v= volume counted

In this case the depth of the hemocytometer slide chambers is 0.1 mm and it covers an area of 1 mm^2. Thus, v= 0.1 mm x1.0 mm^2 = 0.1 mm^3/ 10^{-4} ml

Therefore c=n/v = n/10^{-4} ml = n x 10^4/ml

3.3.4.3 Agar diffusion assay

An agar diffusion method described in (Mahlo *et al*., 2010) was used with some modifications. This technique employs diffusion principles to dispense a component around media in petri dish. In this case, 1 ml of suspended fungal spores was immediately pipetted and spread unto solidified PDA in a 90 mm petri dish and allowed to dry. Wells (6 mm in diameter) were aseptically made on the agar using a well borer and the sample (extract) pipetted into the wells. Plates were then incubated at room temperature for 7 days. The main concept of this technique is the diffusion of sample into the surrounding area of the agar. Extract with active antifungal components exhibits a clear zone of fungal growth inhibition around the well. The zones of inhibitions were measured, and reported as the mean (n=3) ± standard deviation with the statistical analysis carried out at 0.05 level of significant using One-way ANOVA Origin 6.0 software. In addition, the % minimum inhibition zone (%MIZ) was calculated according to the following equation (Mamba *et al*., 2010).

% minumun inhibition zone (MIZ) = (πr^2/ πR^2)X 100

where r = $r_2 - r_1$; r_2: radius of zone of inhibition of the control (AmB),

r_1: radius of zone of inhibition of the test compound and R: radius of petri dish

3.3.4.4 Microplate dilution assay

The minimum inhibitory concentration (MIC) of *P. africanum* was determined using the microplate dilution assay described by from Eloff (1998) and Amiguet *et al*. (2006) with some modifications. The same fungal inoculum quantification method used for the well diffusion and the bioautography assay was employed for this assay. First, a 100 µl of water was added to each well in a 96 well plate. An equal volume of extract dissolved in dimethyl sulfoxide (DMSO) (16 mg/ml) was then added to wells containing amphotericin B (positive control) and water with DMSO (negative control). A 9 fold serial dilution of the sample was made such that the concentration was diluted from the first well (16 mg/ml) until final well with 0.031 mg/ml. A 100 µl of fungal inoculum (in PDA broth) was then added into each well, and plates incubated at 37°C and 5% humidified incubator for 16 and 24 hrs intervals.

After incubation, 40 µl of 0.2 mg/ml INT was added in each well and the plates were again incubated at room temperature for 6 hrs. The indicator INT is principally reduced to a red colour in the presence of viable cells. Thus, when antifungal activity is present, non-viable cells will not show any colour development. The MIC recorded is the lowest concentration of extract that was able to inhibit fungal growth and showed no red colour development.

3.3.5 Electron paramagnetic resonance (EPR) studies

3.3.5.1 Measurement of free radical activity

The free radical activity of the plant species (*M. longifolia*, *L. leonurus* and *P. africanum*) were measured using EPR spectroscopy according to the methods described by Zheleva *et al*. (2011). The EPR studies on powdered and ethanol solutions of each extract before and after 2 hrs UV irradiation were determined using the EPR settings in Table 3.3 below.

Table 3.3: The EPR settings

Settings	Powered sample	Aqueous sample	Ethanol sample
gain	2×10^2	1×10^5	5.02×10^3
Microwave power	0.645 mW	6.494 mW	0.645 mW
Center field	3513.5 G	3514 G	3516 G
Time constant	163.840 ms	163.840 ms	163.680 ms
Sweep time	16.384 s	16.384 s	167.94 s
Modulation amplitude	10.00 G	10.00 G	5.00 G
Number of scans per sample	1	5	1

3.3.5.2 The % DPPH radical scavenging capacity

The radical scavenging capacity of the extracts to donate a hydrogen atom or electron and scavenge the DPPH radical is based on the reduction of a ethanol solution by DPPH (Santos *et al*., 2009). To each 250 µl ethanol DPPH stock solution, three volumes (10, 20 and 30 µl) of each sample extract dissolved in 98% ethanol, were added each sonicated for 5 min in a sonicator water bath (Elmasonic PH750 EL). After 10 min incubation, the mixtures were transferred into capillary tubes. The tubes were sealed, placed in a standard EPR quartz tube (3 mm), and finally into the EPR cavity. The control consisted of 250 µl of DPPH ethanol solution and 10, 20 and 30 µl of ethanol. The % DPPH radicals scavenged by each extract were measured and calculated using the equation below (Zheleva *et al*., 2011).

Scavenged DPPH radical (%) = $[(I_0\text{-}I)/I_0] \times 100\%$

I_0 = integral intensity of the DPPH signal of the control sample

I = integral intensity of the DPPH signal after addition sample to the control

3.3.5.3 Effect of high energy ultrasonic waves on the % DPPH radical scavenging capacity of *P. africanum*

The effect of incubation time and high energy ultrasonic waves on the DPPH radical scavenging capacity of *P. africanum* was determined, since it had the highest % scavenging capacity when compared to *M. longifolia* and *L. leonurus*. The samples were divided into 2 groups. The first consisted of 10, 20, and 30 µl of 0.1% *P. africanum* and the second group 10, 20, 30, 50, and 100 µl of 0.0125% *P. africanum* stock. Each sample was added separately in 250 µl of DPPH ethanol solution. The mixtures were incubated for 2 hrs intervals in the dark at room temperature. When the time elapsed the samples were sonicated for 5 min and their free radical scavenging capacities measured. The % DPPH radicals scavenged by each sample were measured and then calculated.

3.3.5.4 Effect of incubation time on % DPPH scavenging activity of *P. africanum* extract

The effect of the incubation time on the radical scavenging abilities of naturally isolated antioxidants from *P. africanum* was determined. After the 2 hrs UV irradiation, mixtures of different concentration of extract plus DPPH were sonicated for 5 min in a sonicator water bath (Elmasonic PH 750 EL). They were then incubated for 10, 20, and 30 min, and 24 hrs in the dark, and the EPR spectra were recorded, and the % DPPH scavenging activity determined.

3.3.5.5 Determination of superoxide dismutase (SOD) activity before and after UV irradiation

The determination of superoxide dismutase (SOD) activity *in vitro* before and after 2 hrs UV irradiation was conducted. The SOD activity was determined as described by Gadjeva *et al*. (2008). A 100 µl sample was mixed with a 1.5 ml kit consisting of 1 mg hypoxanthine/xanthinoxidase, 14 mg nitro blue tetrasole, 40 mg EDTA, 100 ml phosphate buffered saline (PBS) and 400 µl xanthioxidase. This system produces the superoxide anion for the reaction. The anion ultimately reduces nitro blue tetrasole (NTB) to formosan, detectable at 560 nm wavelength. The results are expressed as units per gram haemoglobin (U/gHb).

3.3.6 EPR *ex vivo* experiments

3.3.6.1 Animal treatment

EPR *ex vivo* studies of Ascorbyl (•Asc) radical levels and ROS production in organ homogenates of mice were also determined. White laboratory healthy mice weighing 31-39 g were used. The mice were housed in polycarbonate cages under controlled conditions of 12 hrs light/dark cycles, temperature of 18-23^{o}C and humidity of 40-60%, with free access to tap water and standard laboratory chow. Experiments were carried out in accordance with European directive 86/609/EEC of 24.11.1986 for protection of animals used in scientific and experimental purposes. The mice were divided into groups (4 mice in each group). For the first group, each mouse was administered with a dose of the sample prepared by dissolving the powdered extract obtained from *P. africanum* in saline solution. The treatment dose was 150 mg/kg. The control group was administered with the solvent. After 3 hrs administration, all

animals in the tested and control group were exsanguinated under light ether anesthesia. The liver and brain were immediately collected, washed in cool saline and the tissue homogenates were then prepared (Karamalakova *et al*., 2010; Zheleva, 2013).

3.3.6.2 •Asc radical and ROS production levels

The •Asc levels in organ homogenates were studied according to Buettner and Jurkiewicz (1993) with modifications made by Zheleva *et al*. (2011). Specimens obtained from liver, kidney, brain and heart were collected in cold saline and processed immediately. Tissue samples were weighed and homogenized in DMSO (10% w/v) and centrifuged at 4000 g, 4 ^{0}C for 10 min. The supernatants were collected and levels of •Asc radicals evaluated by EPR spectroscopy.

Preparation of the homogenates and EPR study of the level of ROS production was performed according to Zheleva *et al*. (2011) and Zheleva (2013). Briefly, about 0.1 g of liver, kidney, brain and heart samples were homogenized after the addition of 1.0 ml of 50 mM solution of the spin-trapping agent, n-tert-butyl-alphaphenylnitrone (PBN), dissolved in DMSO. After centrifugation, 0.4 ml supernatant of the homogenized tissue was placed in a quartz tube and stored in liquid nitrogen for EPR measurement. The EPR settings used for determining Ascorbate radical and ROS production levels are presented in Table 3.4.

Table 3.4: EPR spectroscopy settings for studies of Ascorbyl and ROS radical levels in organ homogenates of mice

Specification	•Asc radical	ROS production
center field (G)	3505	3503
sweep width (G)	30	100
microwave power (mW)	12.70	12.83
receiver gain	1 x 10^4	1 x 10^6
mod. Amplitude (G)	5.00	5.00
time constant (ms)	327.68	327.68
sweep time (Secs)	82.94	81.92
scans per sample	1	5

3.3.6.3 Statistical analysis

Statistical analysis was performed with Statistica 6.1 StaSoft Inc. (Grigorov *et al.,* 2014) and results were expressed as means± standard error (SE). Statistical significance was determined by the Student's t-test and $p \leq 0.05$ values were considered probability level.

3.3.7 Cytotoxicity studies

3.3.7.1 Isolation and purification of lymphocytes

Blood from a healthy donor was placed into 3x5 ml heparin tubes with gentle mixing to avoid clotting. The blood was then mixed with RPMI- media, mixture over-layed on Histopaque and centrifuged at 300 g for 30 min. Thereafter, the interface layer consisting of mononuclear cells was pipetted into tubes and washed 3x with RPMI; each wash consisted of a 5 min centrifugation at 300 g. The pelleted cells were then re-suspended in 10 ml complete culture media (CCM) consisting of: 5 ml fecal bovine serum (FBS), 500 µl penicillin and streptomycin and 45 ml RPMI. Each 10 ml (CCM and cell suspension) was transferred into plastic tissue culture bottles and incubated at 37°C in 5% CO_2 humidified incubator for 24 hrs. The paleness/cloudiness of the media indicates cell growth (Mwanza *et al*., 2009).

3.3.7.2 Cell enumeration

A blood sample of 100 ul of cell suspension was taken before incubation. This sample was mixed with an equal volume of 0.2% trypan blue solution in an Eppendorf tube and incubated at room temperature for 5 min. After which 20 µl of the mixture was pipetted to both chambers of a Hemocytometer covered with a glass cover slip. The cells were viewed under microscope to note viable cells, which are not stained and dead cells which take up the dye appeared blue. Cell viability was determined according to Mwanza *et al*. (2009) as:

% Viability= (viable cell counted/ total number of cells) x 100

3.3.7.3 Methyl thiazol tetrazolium (MTT) assay

After 24 hrs incubation elapsed, cells were transferred into 96-wells culture plates. The 96-wells plates consisted of culture media RPMI, phytohaematoglutinin (PHA) a cell stimulant, cells, plant extracts, and mycotoxins, i.e., OTA and FB_1 dissolved in O.1% dimethylsulphoxide (DMSO) and phosphate buffered saline (PBS). The negative control consisted of all these components with the exception of cells while the positive control consisted of all except for mycotoxin or plant extract. Three different concentration of plant extract (2, 1, and 0.1 mg/ml) and mycotoxins (20, 40, and 80 ug/ml) were used each in triplicates. The number of cells per treatment group was keep constant to ensure reproducibility. After the addition of all these components, the plates were then incubated in a 5% CO_2 humidified incubator at 37°C for 24, 48, and 72 hrs (Mwanza *et al*., 2009).

Cytotoxicity experiment was performed after the incubation time lapsed for each of the three separate times by the addition of 20 ul of MTT solution with gently mixing. MTT solution was prepared by dissolving MTT salt in 0.14 M PBS (pH 7.4) and filtering through 0.22 uM pore sized pressure filter. After the addition of MTT, plates were re-incubated for 4 hrs and then 30 ul DMSO added to each well and incubated further for 2 hrs. This incubation enables the formazan crystals to form. The optical density (OD) was read using a microplate reader at 540 and 620 nm. Cell viability was determined as

% viability = (ODM/ODN) X100

Where ODM is the OD value of the mycotoxin-treated cell and ODN is the OD value of the control

3.3.7.4 Statistical analysis

The results were expressed as means (n=3) ± standard deviation, statistical signand $p \leq 0.05$ values were considered probability level using the one way analysis of variance ANOVA followed by t-test using Origin 6.0 software.

CHAPTER FOUR

4. RESULTS

4.1 Introduction

Extracts of leaves of *Mentha longifolia*, *Leonotis leonurus* and the stem bark of *Piptadeniastrium africanum* were screened for their phytochemical compositions using thin layer chromatography (TLC), ultraviolet visible spectroscopy (UV-Vis), fourier transform-infrared absorption spectroscopy (FT-IR) and 2 dimensional time of flight mass spectroscopy gas chromatography (2D GCxGC-TOF/MS). The biological assays evaluated the antifungal activity of each extract using agar diffusion and microplate dilution assay. The electron paramagnetic resonance (EPR) spectroscopy studies were conducted to determine the antioxidant activity of each extract using DPPH before and after UV irradiation and the superoxide dismutase (SOD) activity. The *ex vivo* experiments evaluated the Ascorbyl (•Asc) radicals and reactive oxygen species (ROS) production levels in organ homogenates of mice administered with *P. africanum* extract. The hepatoprotective potential of the plant extracts was determined against ochratoxin A (OTA) and fumonisin B_1 (FB_1) using MTT assay. The results of each of these assays are presented in this Chapter.

4.2 Sequential solvent extraction efficacy

The quantities of crude extracts were established from 10 g of each plant material after sequential solvent extraction using the different solvents. Generally, methanol was the extraction solvent that gave the highest yield of extract from all three plants with 8, 7.26, and 6.6 % yield for *M. longifolia*, *L. leonurus*, and *P. africanum,* respectively. On the other hand, hexane obtained the least yields of 1.15, 1.1, and 0.3 % from *M. longifolia*, *L. leonurus*, and *P. africanum*. The percentage yields of each plant material are presented in Figure 4.1 (supplementary data presented in Table A1.1, Appendix I).

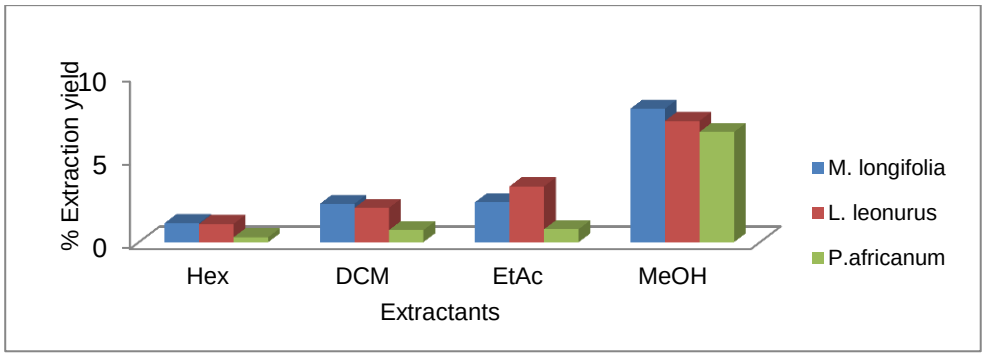


Figure 4.1: Percentage extraction yeild of *M. longifolia*, *L. leonurus*, and *P. africanum* extracted with increasing polarity index solvents hexane (Hex), dichloromethane (DCM), ethyl acetate (EtAc) and methanol (MeOH).

4.3. Phytochemical analyses

4.3.1 Chromatographic profiling of the herbal crude extracts

Thin layer chromatography was performed to separate some of the major compounds present in the plant crude extracts for visualization for further assays or further fractionation. The chromatograms below show the separation profile of *M. longifolia* hexane (Hex), dichloromethane (DCM), etylacetate (EtAc), and methanol (MeOH) extracts from the left to right lanes, developed in a) ethyl acetate: methanol: water 10:1.35:1 (EMW); b) benzene: ethanol: ammonium hydroxide 18:2:02 (BEA); and c) chloroform: ethyl acetate: formic acid 10:8:2 (CEF). In this case the solvent sytem consisting of benzene: ethanol: ammonium hydroxide (BEA) represented in (Figure 4.2) showed better separation and therefore the prefered solvent with the ability to separate some of the compounds in the plant extracts.

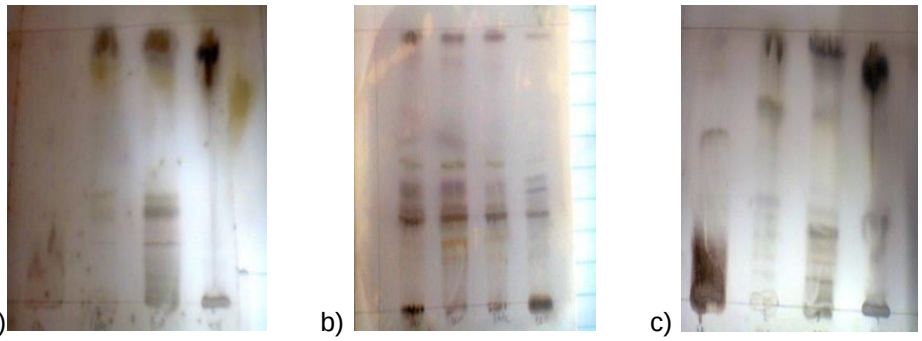


Figure 4.2: TLC chromatograms of *M. longifolia* extracted in (Hex, DCM, EtAc and MeOH) from left to right lanes, developed in solvents a) Ethyl acetate: methanol: water (EMW); b) benzene: ethanol: ammonium hydroxide (BEA); and c) Chloroform: ethyl acetate: formic acid (CEF). All were sprayed with vanillin sulphuric acid for visualization

4.3.2 UV-Vis spectroscopy

The UV absorbance measured in the range of 200-800 nm for each plant extract exhibited characteristic peaks representing the presence of phenolic compounds and their derivatives. The results in Table 4.1 displays three of the highest peak-pick wavelength λ (nm) and absorbance (Abs) recorded for each extract.

Table 4.1: The highest peak-pick absorbance recorded for each plant extract

Sample	Peak i		Peak ii		Peak iii	
	λ (nm)	Abs	λ (nm)	Abs	λ (nm)	Abs
M. longifolia	245	0.049	294	0.32	378	0.077
L. leonurus	295	0.669	305	0.731	412	0.139
P. africanum	209	0.047	300	1.587	776	0.013

4.3.3 Fourier transform-infrared absorption spectroscopy (FT-IR)

The FT-IR analytical data from the methanol extracts of the three plants confirmed the presence of a number of compounds with major peaks. The spectra displayed some prominent absorptions, i.e., a band centered at 3411, 3456 and 3314 cm^{-1} due to the presence of O-H and H-bonded alcohols and phenols, the C-H bond frequency

at 2928, 2923 cm^{-1} assigned to the presence of alkanes. A strong absorption exhibited at 1650 cm^{-1} is assigned to C=C alkenes. The C-O absorption band due to alcohols, esters and carboxylic acids functional groups appeared at 1159 and 1195 cm^{-1}. The spectra recorded for each plant extract are displayed in Figure 4.3 a), b) and c) below, these were assigned to functional groups as presented in Table 4.2.

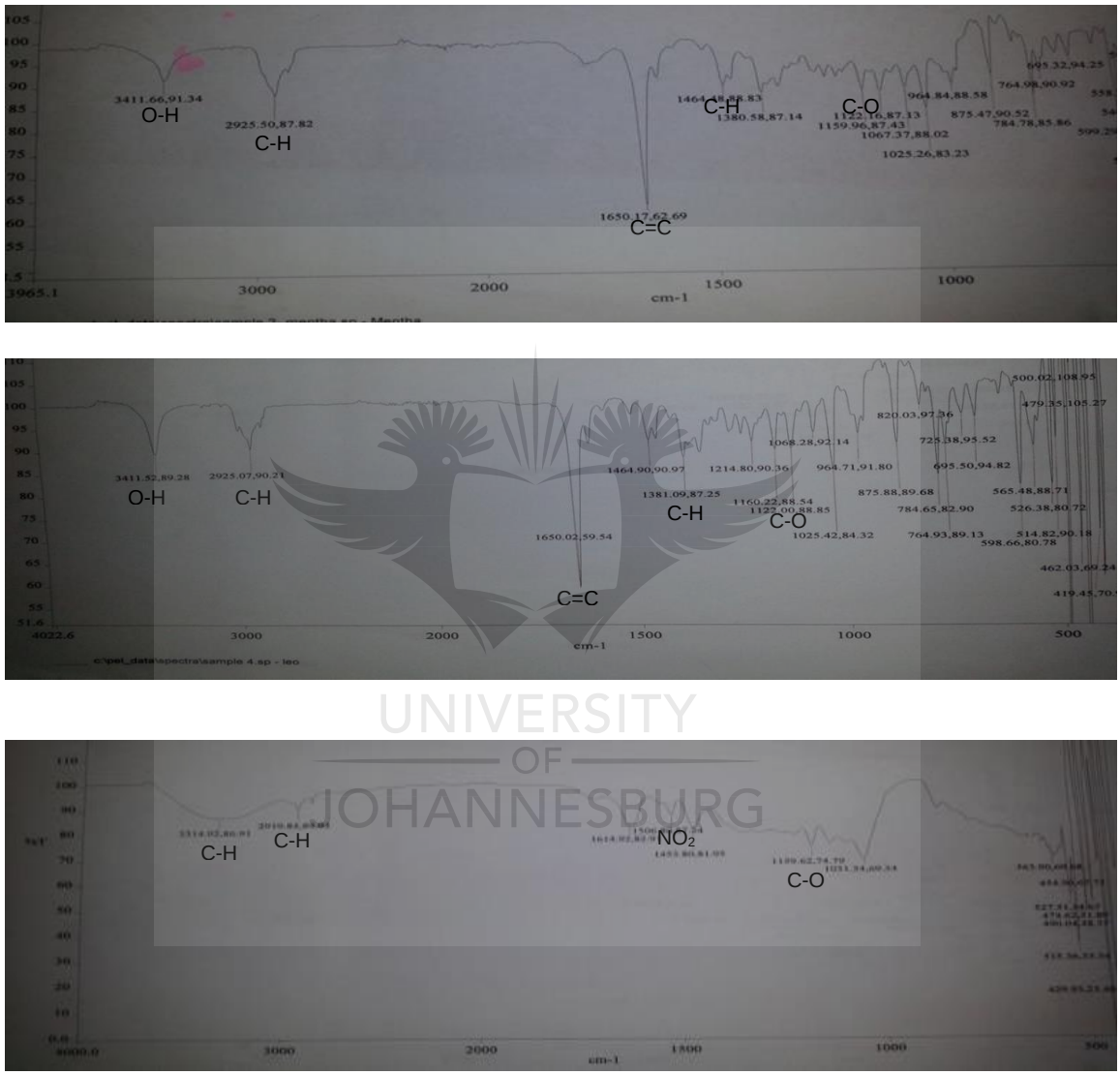


Figure 4.3: FT-IR spectra of a) *M. longifolia* b) *L. leonurus*, and c) *P. africanum* methanol extracts

Table 4.2: Spectral peak values and functional groups obtained from *M. longifolia*, *L. leonurus* and *P. africanum* methanol extracts identified by FT-IR spectroscopy

M. longifolia

Frequency (cm^{-1})	Bond type	Functional groups
3411.66	O-H H-bonded	Alcohols and phenols
2925.50	C-H asy/sym CH_2	Alkanes
1650.17	C=C	Alkenes
1464.48	C-H	Alkanes
1380.58	C-H	Alkanes
1159.96	C-O	Alcohols, ethers, carboxylic acids & esters
964.71-725.38	C-H	Alkanes
695.50	C-H	Alkanes

L. leonurus

Frequency (cm^{-1})	Bond type	Functional groups
3456.34	O-H H-bonded	Alcohols & phenols
2923.32	C-H asy/sym CH_2	Alkanes
2500.79	O-H stretch	Carboxylic acids
2161.18	C≡C	Alkynes
1732.47	C=O	Aldehydes, ketones, carboxylic acids & esters
1659.95	C=C	Alkenes
1462.90	C-H_2 bend	Alkanes
1376.15	C-H	Alkanes
1198.53-10.65	C-O	Alcohols, esters & carboxylic acids

P. africanum

Frequency (cm^{-1})	Bond type	Functional groups
3314.92	C-H or O-H	Acetylic alkyne, alcohols & phenols
2919.84	C-H asy/sym CH_2	Alkanes
1614.92	C=C	Alkenes
1506.80	NO_2	Nitro compounds
1453.80	C-H	Alkanes
1159.62	C-O	Alcohols, ethers, carboxylic acids & esters
1031.34	CH_2 stretch	Cyclohexane ring vibrations

4.3.4 2D GCxGC-TOF/MS

A two dimensional gas chromatography time of flight mass spectrometry (2D GC×GC–TOF/MS) was performed to identify compounds present in methanol extracts of the three plants. A total of 6 per plant extract and 12 co-occurring compounds selected on the basis of their medicinal properties including antioxidant, anti-inflammatory, antifungal, antimicrobial, anticarcinogenic etc. (Tables A1.3, A1.4, A1.5 and A1.6 in Appendice I), and high probability values (similarity indexes above 700) were identified and their structures and mass spectra presented in Figures 4.4, 4.5, 4.6 and 4.7 below. The most abundantly occurring 24/25 compounds in each extract are presented in Tables 4.3, 4.4 and 4.5. The most abundantly occurring compounds with 70% and above similarity within each plant extract are tabulated in Tables 4.3, 4.4, and 4.5. The highlighted compounds have important medicinal properties (Tables A1.3-A1.6 in Appendix I).

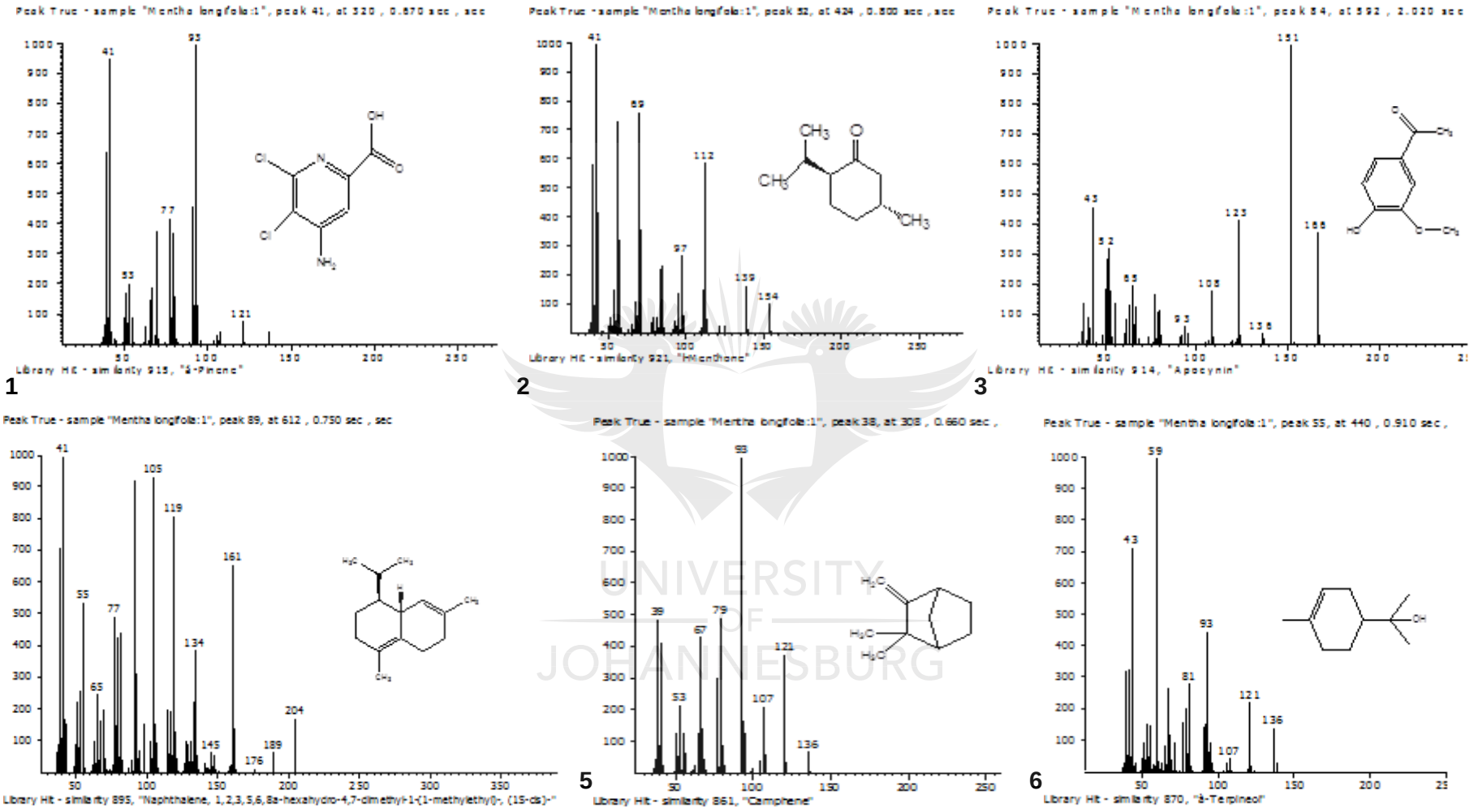


Figure 4.4: The mass spectra of **1:** á-Pinene; **2**: l-Menthone; **3**: Apocynin; **4**: Naphthalene, 1, 2, 3, 5, 6, 8a-hexahydro-4, 7-dimethyl-1-(1-methylethyl)-, (1S-cis)-; **5**: Camphene; **6**: à-Terpineol identified in methanol leaves extract of *M. longifolia*

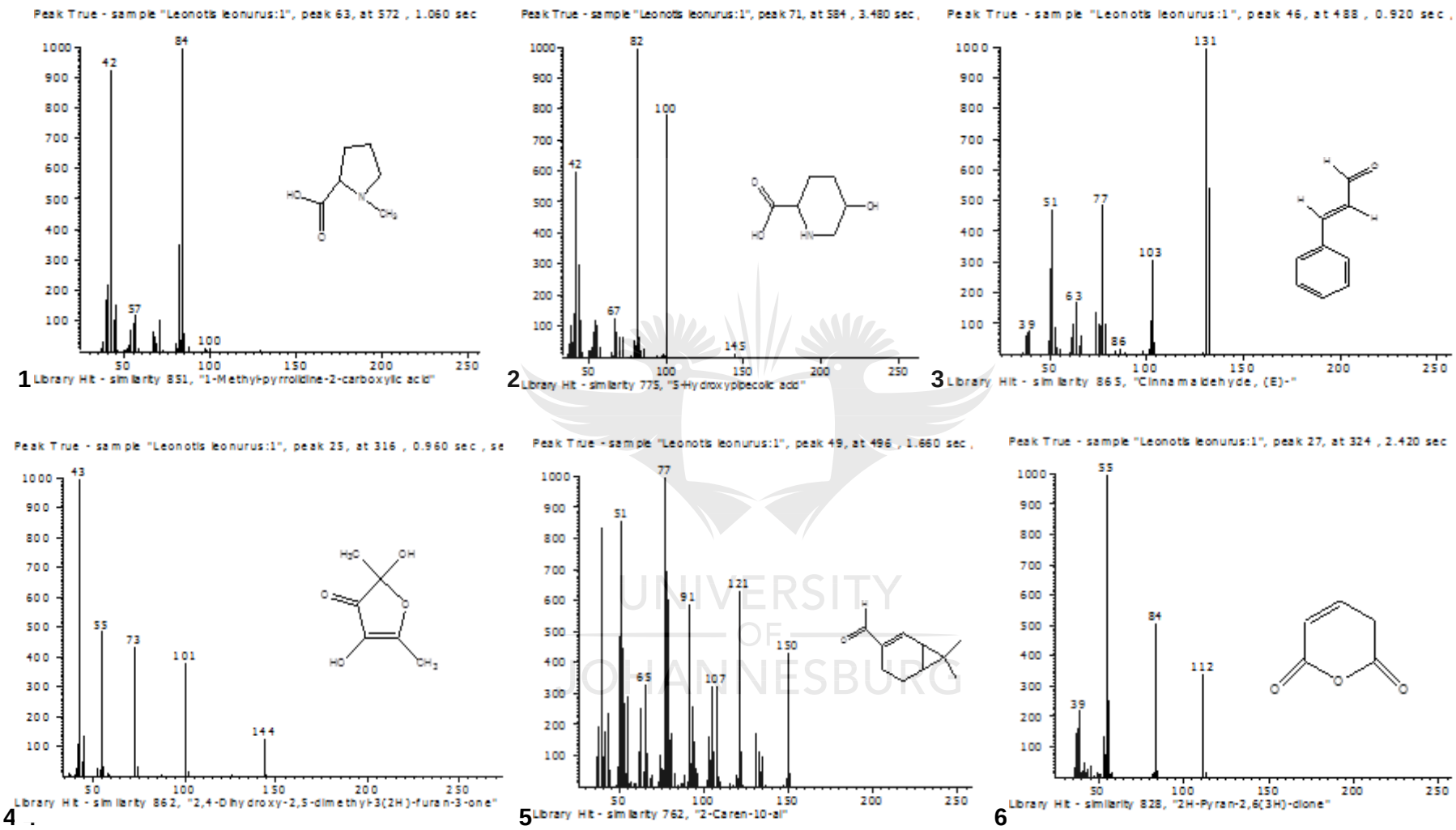


Figure 4.5: The mass spectra of **1**: 1-Methyl-pyrrolidine-2-carboxylic acid; **2**: 5-Hydroxypipecolic acid, **3**: Cinnamaldehyde, (E)-; **4**: 2,4 Dihydroxy-2,5-dimethyl-3(2H)-furan-3-one; **5**: 2-Caren-10-al; and **6**: 2H-Pyran-2,6(3H)-dione, identified in methanol leaves extract of *L. leonurus*

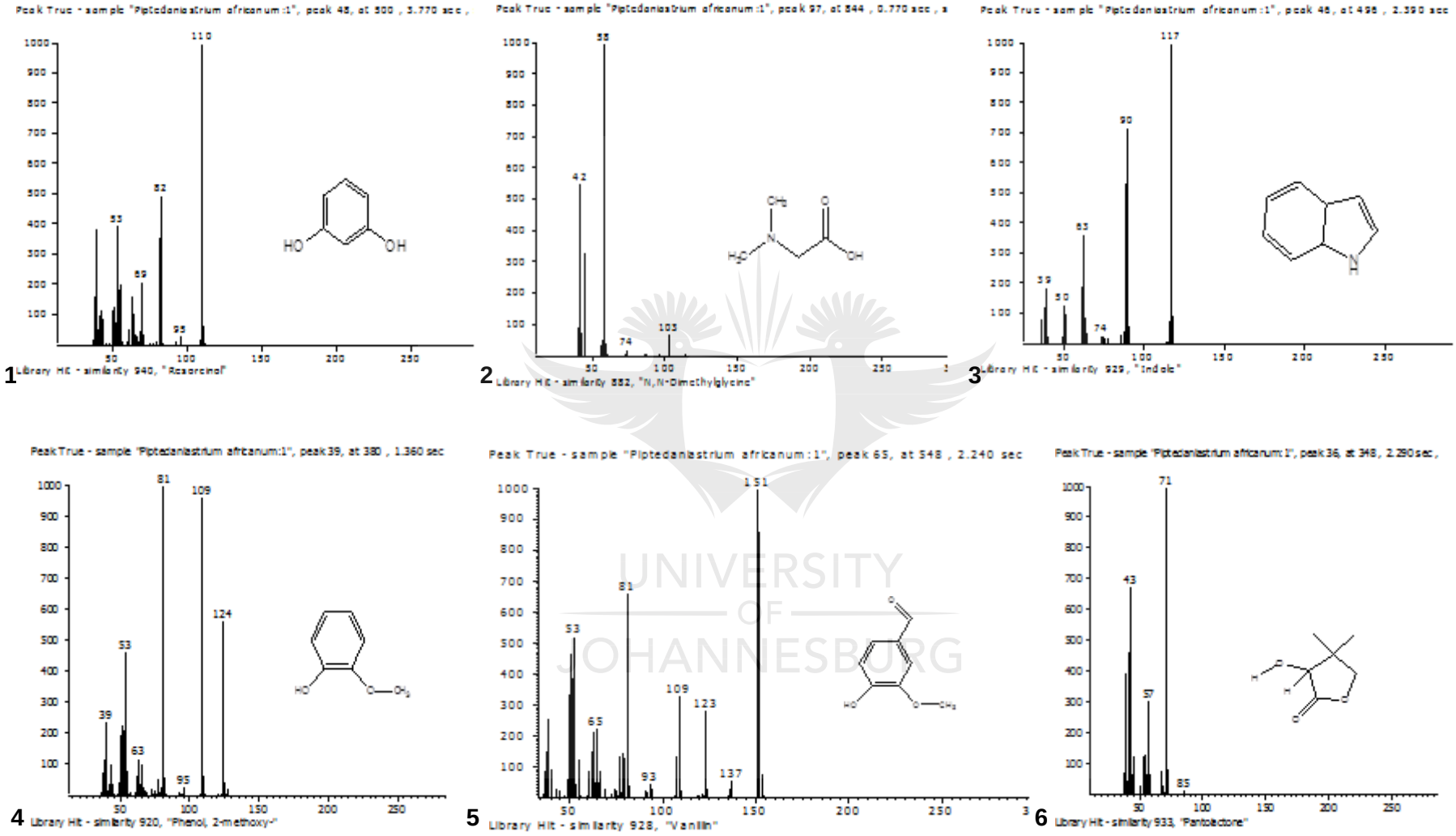
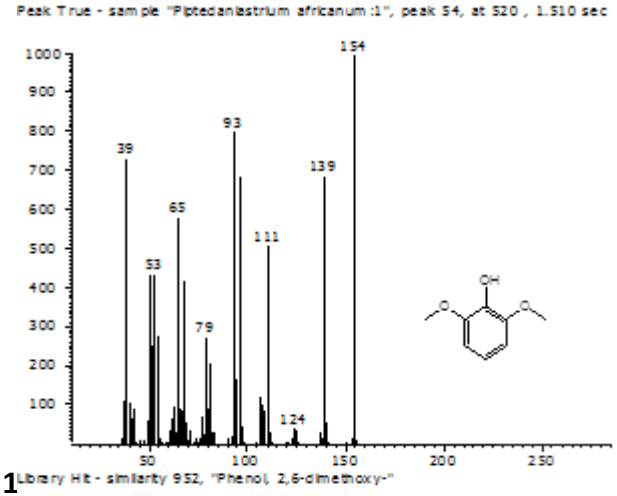
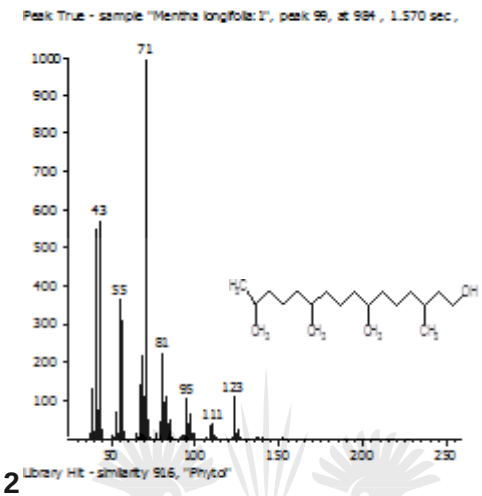


Figure 4.6: The mass spectra of: **1**: Resorcinol; **2**: N,N-Dimethylglycine; **3**: Indole; **4**: Phenol, 2,6-dimethoxy-4-(2-propenyl)-; **5**: Vanillin; and **6**: Pantolactone, identified in methanol sterm bark of *P. africanum* extracts



1



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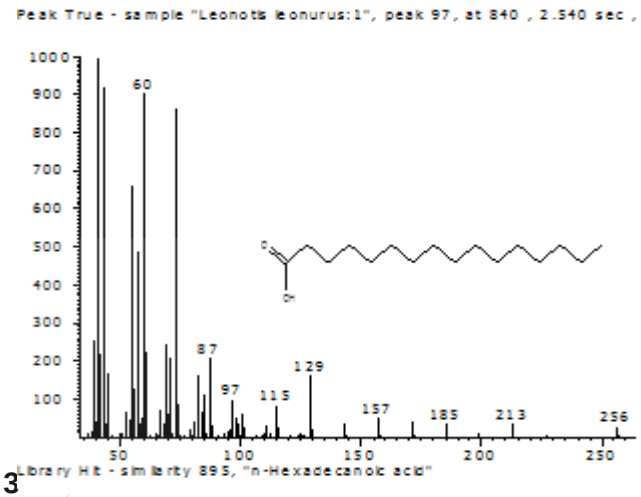

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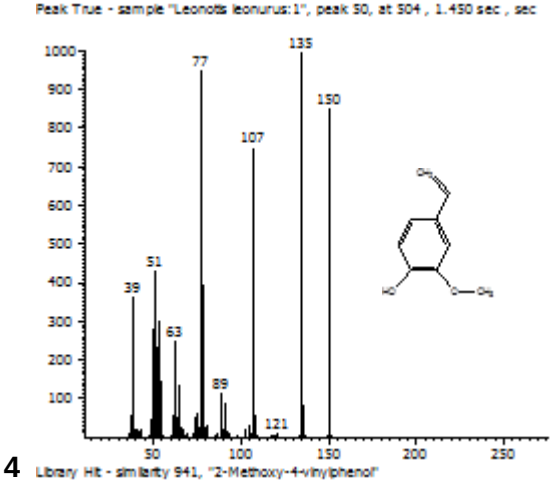

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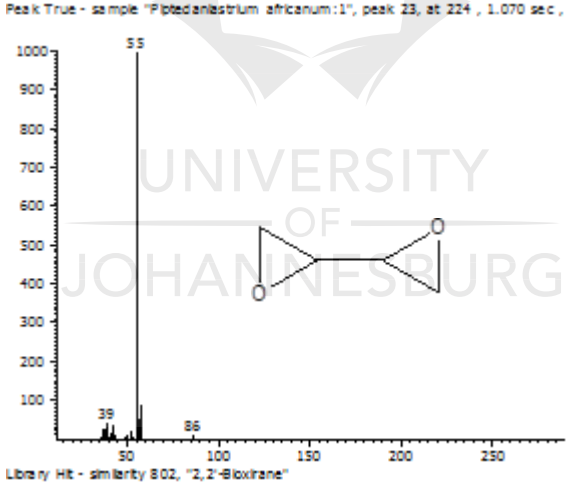

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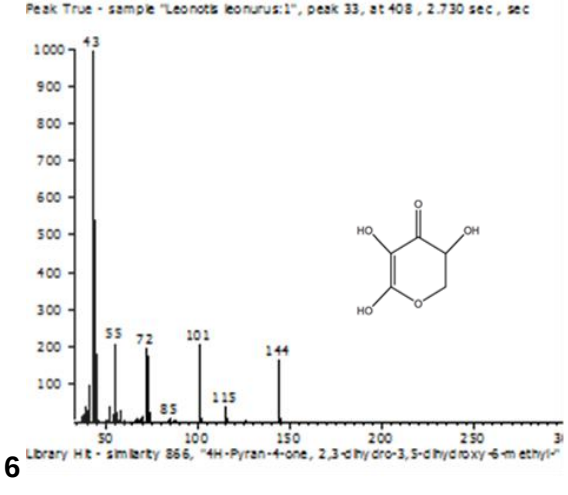

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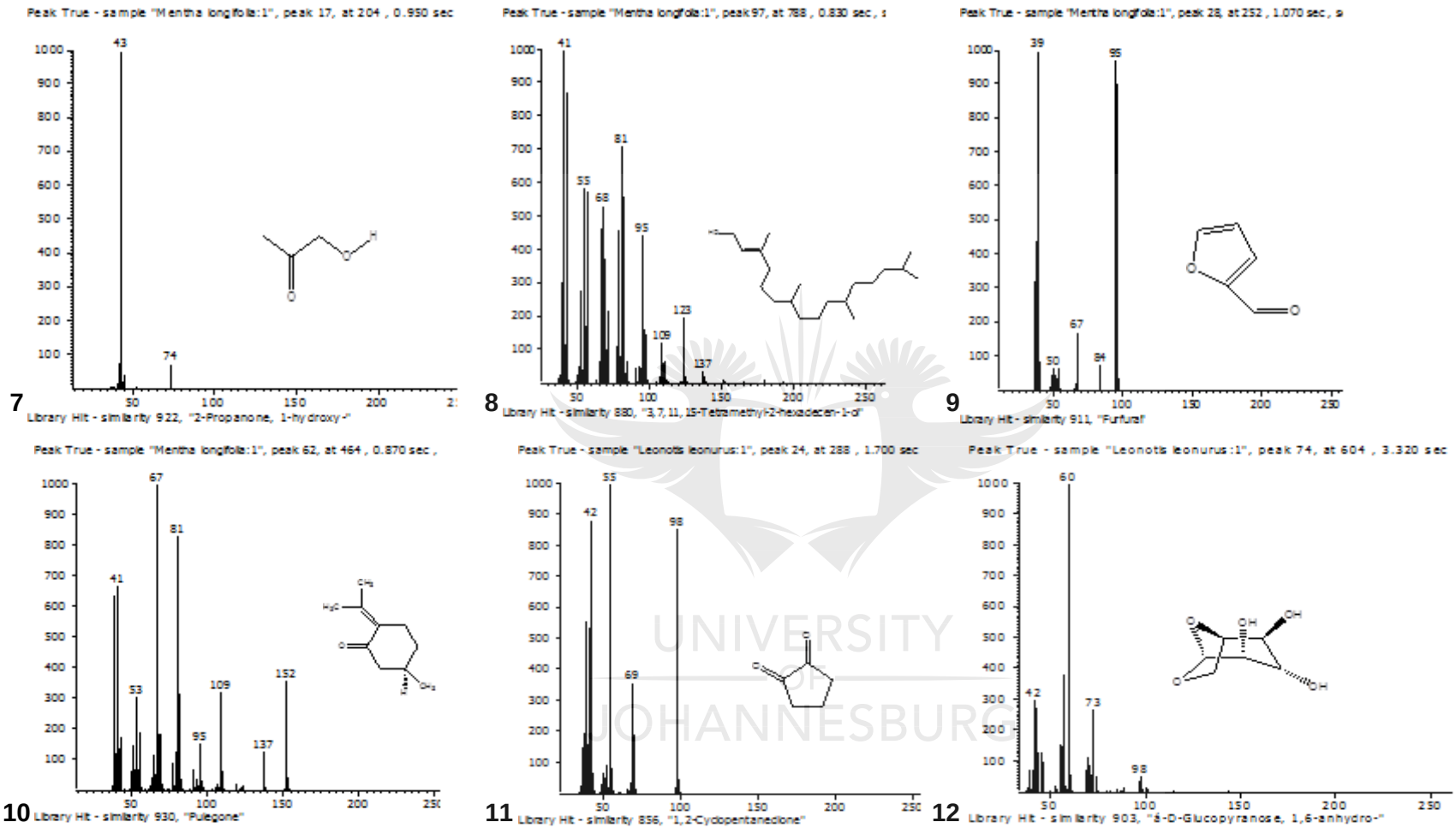


Figure 4.7: Mass spectra of :**1**: Phenol, 2,6-dimethoxy[a b c]; **2**: Phytol [a b]; **3**: n-Hexadecanoic acid [a b c]; **4**: 2-Methoxy-4-vinylphenol [a b c]; **5**: 2,2'-Bioxirane [b c]; **6**: 4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-[a b c]; **7**: 1,2-Cyclopentanedione [a b]; **8**: 3,7,11,15-Tetramethyl-2-hexadecen-1-ol [a b]; **9**: Furfural [a c]; **10**: Pulegone [a b]; **11**: 2-Propanone,1-hydroxy [a b c]; and **12**: á-D-Glucopyranose, 1,6-anhydro- [a b], present in **a**: *M. longifolia*; **b**: *L. leonurus*; and **c**: *P. africanum*

Table 4.3: The most abundantly occurring compounds in *M. longifolia* methanol leaves extract

Peak no.	R.T. (s)	Compound Name	% Peak Area	Weight	Similarity
17	204	2-Propanone, 1-hydroxy-	33.6	74	922
80	580	Sucrose	13.05	342	725
26	248	Acetic acid	8.4	60	973
21	224	Acetic anhydride	7.05	102	927
12	192	Formic acid	3.04	46	990
35	280	Dihydroxyacetone	2.45	90	880
15	200	Hydrogen azide	2.14	43	848
49	412	Glycerin	1.03	92	777
57	488	2-Methylbutanoic anhydride	0.93	186	789
78	444	Benzofuran, 2,3-dihydro-	0.86	120	856
46	384	Cyclopropyl carbinol	0.69	72	825
30	260	Ethanethioic acid, S-(dihydro-2,5-dioxo-3-furanyl) ester	0.66	174	741
3	176	Carbamic acid, monoammonium salt	0.53	78	999
61	464	1,2,3-Propanetriol, 1-acetate	0.45	134	755
54	432	Bicyclo[2.2.1]heptan-2-ol, 1,7,7-trimethyl-, (1S-endo)-	0.4	154	929
100	1020	9,12,15-Octadecatrienoic acid, (Z,Z,Z)-	0.39	278	893
40	316	2,4-Dihydroxy-2,5-dimethyl-3(2H)-furan-3-one	0.36	144	881
70	**504**	**2-Methoxy-4-vinylphenol**	**0.36**	**150**	**939**
36	**288**	**1,2-Cyclopentanedione**	**0.35**	**98**	**854**
29	256	2-Furanmethanol	0.34	98	956
28	**252**	**Furfural**	**0.33**	**96**	**911**
78	573	Benzaldehyde, 2-hydroxy-4-methyl-	0.33	136	730
95	760	1-Octadecyne	0.3	250	837
51	424	Benzofuran, 4,5,6,7-tetrahydro-3,6-dimethyl-	0.23	150	869

Key: R.T. = retention time

Table 4.4: The most abundantly occurring compounds in *L. leonurus* methanol leaves extract

Peak no.	R.T. (s)	Compound Name	% Peak Area	Weight	Similarity
8	188	Acetic acid	13	60	965
14	224	Acetic anhydride	10.1	102	907
69	580	Sucrose	8.99	342	724
41	**456**	**1-Methyl-pyrrolidine-2-carboxylic acid**	**8.66**	**129**	**888**
13	**220**	**2,2'-Bioxirane**	**5.34**	**86**	**800**
18	252	Glyceraldehyde	5.12	90	915
22	280	Dihydroxyacetone	4.47	90	881
46	**488**	**Cinnamaldehyde, (E)-**	**3.00**	**132**	**865**
99	856	Bicyclo[6.1.0]nonane, 9-(1-methylethylidene)-	2.33	164	778
5	184	Methyl glyoxal	2.33	72	767
9	192	Acetaldehyde, hydroxy-	1.73	60	872
71	584	5-Hydroxypipecolic acid	1.66	145	775
39	448	Benzofuran, 2,3-dihydro-	1.53	120	852
26	324	2-Hydroxy-gamma-butyrolactone	1.51	102	838
35	416	Glycerin	1.47	92	806
1	172	Nitrous oxide	1.36	44	999
100	**988**	**Phytol**	**1.16**	**296**	**916**
24	**288**	**1,2-Cyclopentanedione**	**0.66**	**98**	**856**
47	488	5-á,8-á-Epoxy-3,5,8,8a-tetrahydro-1H-2-benzopyran	0.54	150	791
92	760	1-Octadecyne	0.49	250	835
53	508	2-Butanone, 4-(dimethylamino)-3-methyl-	0.43	129	882
52	508	2(5H)-Furanone, 5-(2-furanylmethyl)-5-methyl-	0.29	178	756
32	400	2-Propanamine, N-methyl-N-nitroso-	0.28	102	713
59	548	7-Oxabicyclo[4.1.0]heptane, 3-oxiranyl-	0.26	140	810

Key: R.T. = retention time

Table 4.5: The most abundantly occurring compounds in *P. africanum* methanol stem bark extract

Peak no.	R.T. (s)	Compound Name	% Peak area	Weight	Similarity
63	**544**	**Resorcinol**	**30.4**	**110**	**925**
12	188	Acetic acid	16.8	60	971
39	380	Phenol, 2-methoxy-	4.65	124	920
83	**604**	**N,N-Dimethylglycine**	**4.25**	**103**	**881**
3	176	Carbamic acid, monoammonium salt	3.13	78	999
100	1012	Cyclotrisiloxane, hexamethyl-	3.12	222	906
15	196	Trichloromethane	3.05	118	956
54	**520**	**Phenol, 2,6-dimethoxy-**	**2.76**	**154**	**952**
95	824	2-Propanone, 1-(dimethylamino)-	1.67	101	837
7	184	Formic acid, ethenyl ester	0.95	72	805
73	568	Methylamine, N,N-dimethyl-	0.76	59	914
23	**224**	**2,2'-Bioxirane**	**0.69**	**86**	**802**
20	208	Aziridine, 1-ethenyl-	0.6	69	843
4	176	Chloromethane	0.57	50	969
32	300	Hexanoic acid	0.44	116	928
80	588	1,7-Octadien-3-ol, 2,6-dimethyl-	0.34	154	728
31	296	2,5-Furandione, dihydro-3-methylene-	0.33	112	893
27	252	Furfural	0.31	96	874
26	244	Ethanedioic acid, dimethyl ester	0.28	118	921
10	184	n-Propyl acetate	0.25	102	747
11	188	Methylene chloride	0.24	84	751
13	192	Acetaldehyde, hydroxy-	0.23	60	871
35	344	1,3-Dioxol-2-one,4,5-dimethyl-	0.22	114	798
43	460	1-Dimethyl(isopropyl)silyloxypropane	0.21	160	702
28	252	Dimethyl Sulfoxide	0.2	78	914

Key: R.T. retention time

4.3.5 Biological assays

4.3.5.1 Agar diffusion assay

The agar diffusion assay was used to determine the antifungal activity of *M. longifolia*, *L. leonurus*, and *P. africanum* against four mycotoxigenic fungi until Day 7 and the results are presented in Table 4.6. Generally, extract of *P. africanum* presented the greatest activity which lasted for 7 days, whereas extract of *M. longifolia* displayed no activity at all against the test fungi. On Day 3, the minimum inhibitory zone of 25 mm was achieved for *P. africanum* when compared to the control, Amphotericin B (AmB) that had 15 mm zone of inhibition against *Aspergillus niger*. The antifungal activity of *P. africanum*, was however, not long-lasting as it reduced over time, i.e., from 25 mm on Day 3 to 11 mm on Day 7. None of the extracts tested exhibited any antifungal activity towards *A. fumigatus* even on Day 3.

Table 4.6: The average zone of fungal growth inhibition measured (mm) for *P. africanum* sample

Average Diameter of measured zone of inhibition (mm)								
	A. flavus		*A. fumigatus*		*A. parasiticus*		*A. niger*	
Day	AmB	Sample	AmB	Sample	AmB	Sample	AmB	Sample
3	20±0.02	15±0.09	0±0.00	0±0.00	18±0.08	15±0.05	15±0.20	25±0.16
5	19±0.04	10±0.04	0±0.00	0±0.00	16±0.04	12±0.20	13±0.04	15±0.40
7	14±0.08	9.9±0.09	0±0.00	0±0.00	15±0.09	10±0.30	13±0.08	11±0.20

The inhibition zones in diameter (mm) expressed as the mean (n=3) ± standard deviation. The means at the 0.05 level are not significantly different according to ANOVA Origin 6.0 software

4.3.5.2 Microplate dilution assay

The extract of *P. africanum* which demonstrated the greatest antifungal activity in both the well diffusion and bioautography assays, was further used to determine the minimum inhibitory concentration (MIC) against fungal growth. The results were recorded and tabulated in Table 4.7. As found, MIC of 2 mg/ml of the extract exhibited some inhibition against *A. flavus* and *A. parasiticus*, meanwhile against *A. ochraceus* and *A. niger*, the MIC recorded was 4 mg/ml.

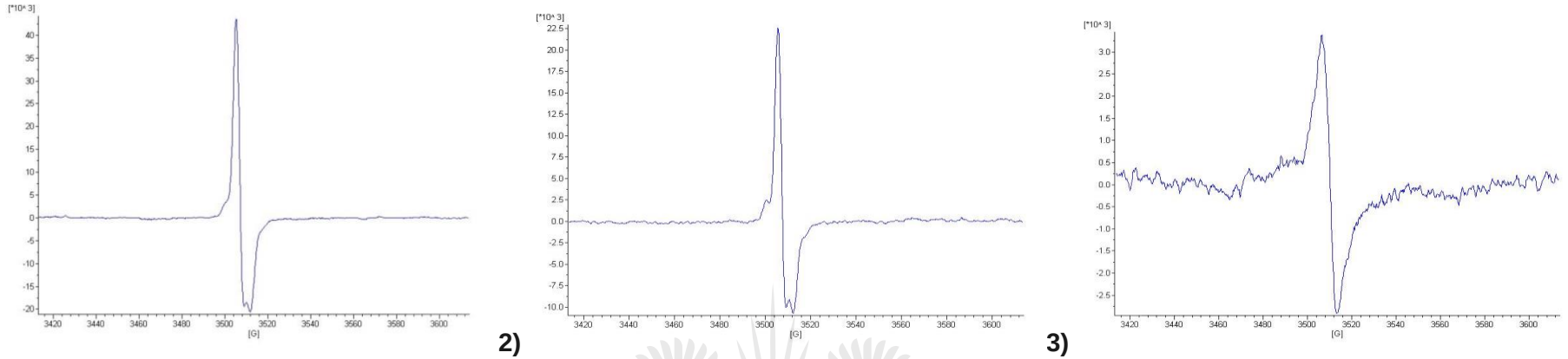
Table 4.7: The MIC values for *P. africanum* stem bark extract

	Sample dilution concentration (mg/ml)									
Fungal species	16	8	4	2	1	0.5	0.25	0.125	0.063	0.031
A. flavus	-	-	-	-	+	+	+	+	+	+
A. ochraceus	-	-	-	+	+	+	+	+	+	+
A. parasiticus	-	-	-	-	+	+	+	+	+	+
A. niger	-	-	-	+	+	+	+	+	+	+

Key: (-) absence of growth/ inhibition and (+) presence of growth

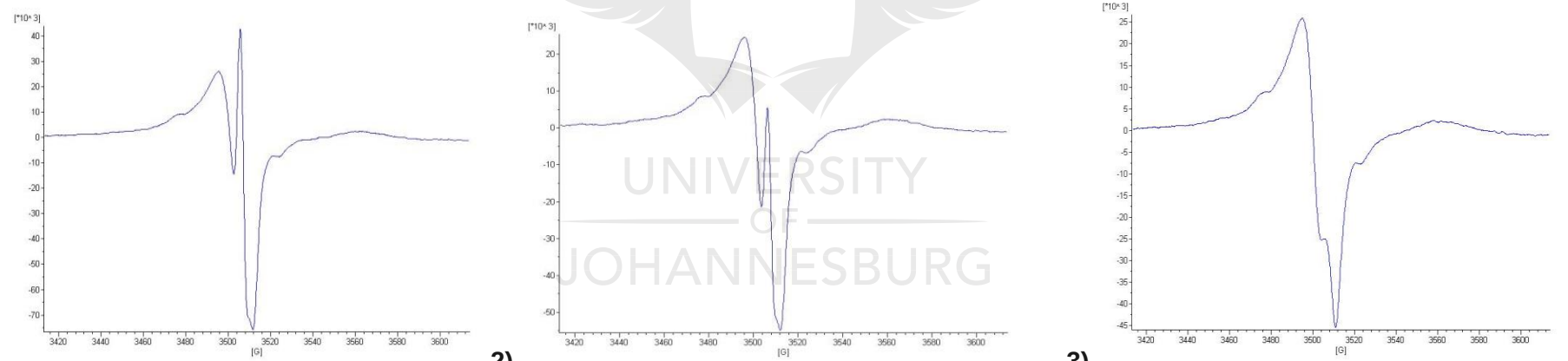
4.3.6 EPR spectroscopy studies of free radical activity

The EPR spectra of samples of the three plants with or without 2 hrs of UV-irradiation were generated using EPR spectroscopy. The free radicals detected in *M. Longifolia*, *L. leonurus*, and *P. africanum* powdered sample using EPR spectroscopy displayed a singlet EPR spectra (Figure 4.8). The g-values recorded for *M. longifolia* before UV irradiation were 2.00637 and 2.01108 on the left (Figure 4.8a:1). After 2 hrs UV irradiation, new radicals were seen on the left with a g-value of 2.01123, 2.00641, and a small radical on the right with a g-value of 2.00451 (Figure 4.8b:1). The free radicals present in *L. leonurus* powdered samples had g-values of 2.00645, 2.00411 on the left and 2.00993 on the right before irradiation (Figure 4.8a:2). After 2 hrs of UV irradiation, the g-values were 2.00637 and 2.01108 on the left (Figure 4.8b:2). The free radicals detected in *P. africanum* powdered extract recorded before irradiation, had g-values of 2.00641 and 2.00418 on the right before irradiation (Figure 4.8a:3). The g-values were 2.01123, a new radical on the left, 2.00641 and 2.00451 on the right after 2 hrs UV irradiation (Figure 4.8b:3).



1) 2) 3)

a) Before UV irradiation



1) 2) 3)

b) After UV irradiation

Figure 4.8: EPR spectra of free radicals detected a) before and b)after UV irradiation of 1: *M. longifolia*; 2: *L. leonurus* and 3: *P. africanum*

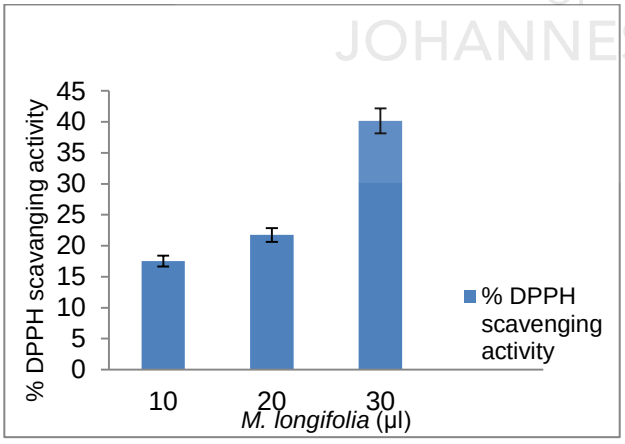
4.3.7 The % DPPH scavenging activity

The leaf extracts of *M. longifolia*, *L. leonurus* and *P. africanum* scavenged DPPH in a concentration dependent manner as seen in Tables A3.1, A3.2, and A3.3 in Appendice III and Figures 4.9 a), 4.10 a), and 4.11 a) below. The methanol extracts at 0.1% concentration (10 µl) of *M. longifolia* had the lowest (17%), followed by *L. leonurus* with a moderate (27%) and *P. africanum* with the highest (99%) DPPH scavenging activity. The 40 and 69% DPPH scavenging activity of the extracts of *M. longifolia* and *L. leonurus*, respectively, both at 0.1% and 30 µl indicate that *L. leonurus* had a more significant activity than *M. longifolia*. The extracts of *P. africanum* were further diluted to 0.125% concentration and still displayed high DPPH scavenging activity. The spectra of selected samples within each plant extract are displayed in the Figures 4.9b), 4.10b), and 4.11b).

4.3.7.1 The % DPPH scavenging activity of *M. longifolia*

The % DPPH scavenging activity of *M. longifolia* recorded was concentration dependent but relatively low, 17, 22, and 40% at 10, 20 and 30 µl, respectively (Figure 4.9a). Despite this, the EPR spectrum with hyperfine nuclear splitting (Figure 4.9b) displayed the presence of free radicals scavenging irrespective of the low scavenging capacity.

a)



b)

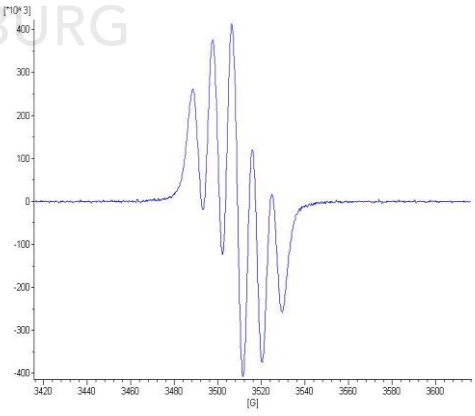


Figure 4.9: a) The % DPPH scavenging activity of *M. Longifolia* (5% error) and b) EPR spectrum of 10 µl *M. longifolia* and 250 µl DPPH

4.3.7.2 The % DPPH scavenging activity of *L. leonurus*

The % DPPH scavenging activity of *L. leonurus* recorded were 27, 69, and 99%, respectively, for 10, 30 and 100 µl, which increased with increasing sample concentration as seen in Figure 4.10a and the EPR spectrum recorded in Figure 4.10b below.

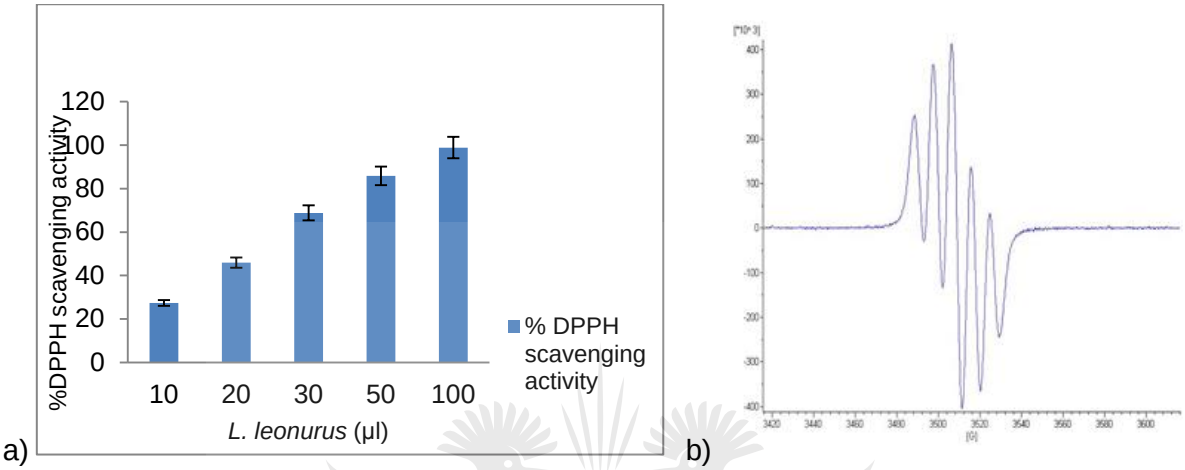
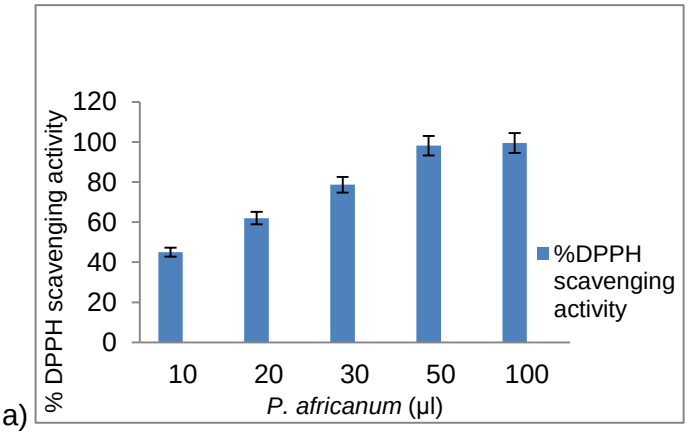


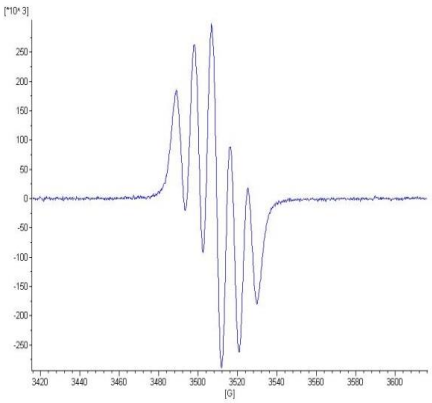
Figure 4.10: a) % DPPH scavenging activity in 0.1% solution (5% error) and b) EPR spectrum of 10 µl of *L. leonurus*

4.3.7.3 The % DPPH scavenging activity of *P. africanum*

The *P. africanum* extract showed superior % DPPH activity of 99 % at 10 µl (Table A3.3 in Appendice III). The concentration was further diluted from 0.1 to 0.0125 % and the antioxidant activity was still considerably high at 45, 62, 79, 98 and 99% respectively, for 10, 20, 30, 50 and 100 µl of extract (Figure 4.11a) which again, was concentration dependent. Figure 4.11b represents the EPR spectra of free radical scavenging activity of components in this extract.



a)



b)

Figure 4.11: a) The % DPPH scavenging activity from 0.125% stock (5% error) and b) EPR spectrum of 10 µl solution of *P. Africanum* extract

4.3.8 The % DPPH radicals scavenged after UV irradiation

4.3.8.1 The % DPPH radicals scavenged after UV irradiation and exposure to high ultrasonic waves of *P. africanum* extract

The trend observed after exposure to 2 hrs UV irradiation and exposure to ultrasonic waves indicated an increase in % DPPH scavenging activity of *P. africanum* extract with increasing concentration as further dilution of the stock solution from 0.1 to 0.125% decreased the % DPPH scavenging capacity. Despite this, increasing the dosage also resulted in an increase in % DPPH scavenging activity (Figure 4.12 and Table A3.4 in Appendix III).

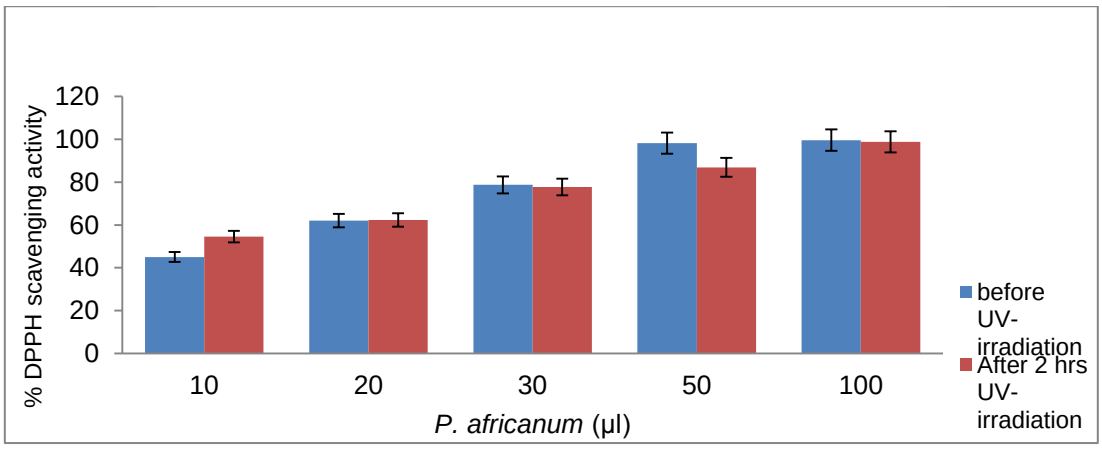


Figure 4.12: The % DPPH scavenging activity before and after 2 hrs UV irradiation and exposure to high ultrasonic waves of *P. africanum* extract

4.3.8.2 The effect of incubation time on the % DPPH scavenging activity before and after UV irradiation

The % DPPH scavenging activity at 0.0125% concentration of *P. africanum* before and after 2 hrs UV irradiation was measured after 10 and 30 min of incubation in the dark. The results displayed in Figure 4.13a and Table A3.5 in Appendix III, showed an increase in the % DPPH scavenging activity after exposure to 2 hrs UV irradiation which increased with increase in incubation time, which was not the case without UV irradiation treatment. The % DPPH scavenged at (10 µl) of *P. africanum* extract increased over time from 8.51% after 2 hrs UV irradiation to 43.51% after 24 hrs irradiation, a wide difference of 35%. A negligible increase in % DPPH was observed when using a volume of 50 µl against the cells. These results are displayed in Figure 4.13b and Table A3.6 in Appendix III.

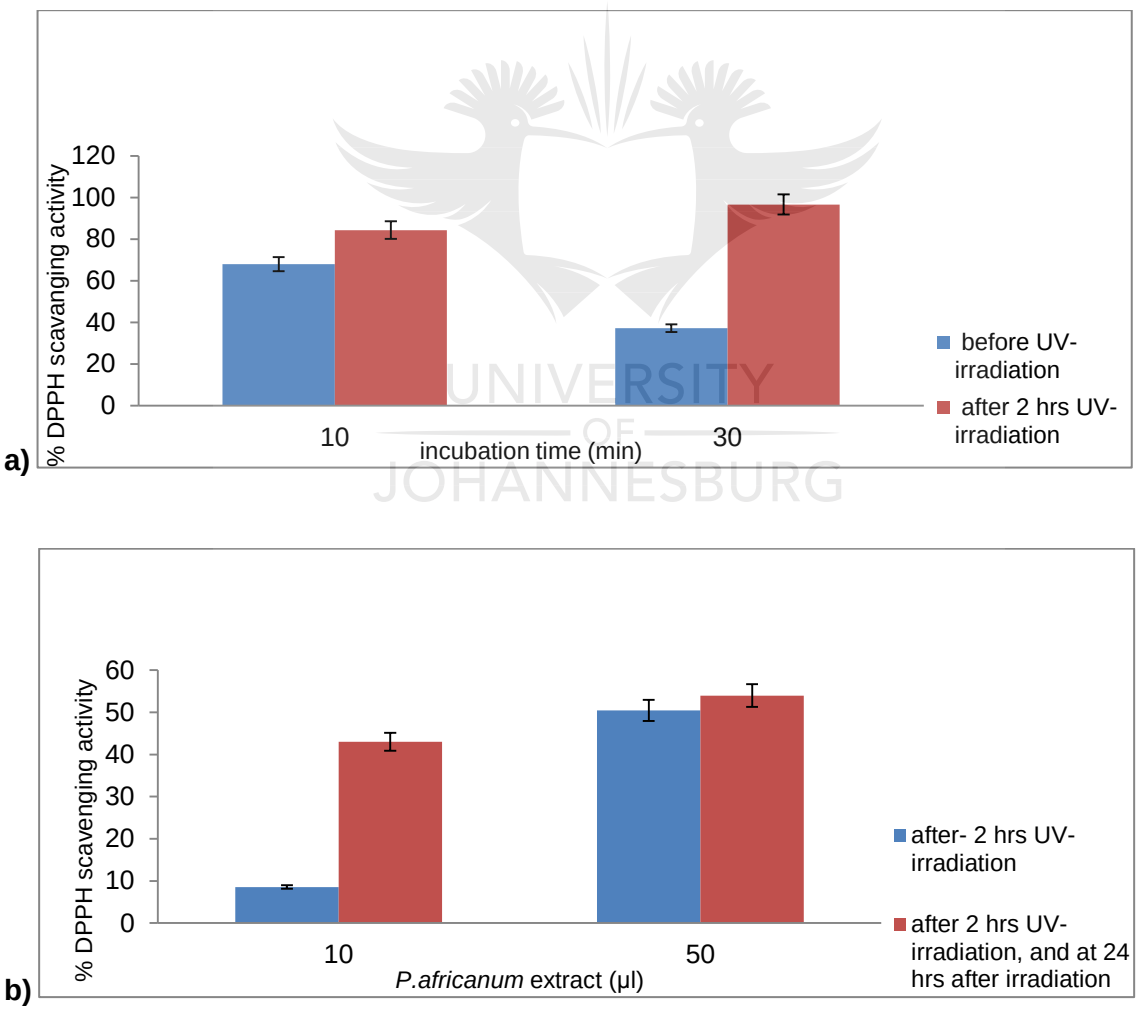


Figure 4.13: The effect of *P. Africanum* before and after 2 hrs UV irradiation at a)10 and 30 min after incubation and b) at 24 hrs after irradiation time

4.3.8.3 The SOD activity (Units/mg) of *P. africanum* before and after UV irradiation measured at 2 and 24 hrs after radiation time

The results of the SOD activity of *P. africanum* revealed that SOD activity of *P. africanum* only before UV irradiation recorded was 12.22 Units/mg, meanwhile no activity was observed after 2 hrs UV irradiation as well as after 24 hrs of incubation.

4.3.9 *Ex-vivo* studies

4.3.9.1 The ascorbly (•Asc) radical levels in organ homogenates of mice

High levels of •Asc radicals were expressed in the organ homogenates with a mean of 0.61415 for *P. africanum* extracts thrice as high as that of the control (0.18305) in the liver homogenates. Also, a mean of a level of 0.9605 •Asc radicals expressed by *P. africanum* extract was more than twice the levels of •Asc radicals in the brain homogenates for the control (0.3375). Figure 4.14 (Table A3.7 in Appendix III) displays the •Asc radicals in both the liver and brain homogenates after exposure to *P. africanum* extract.

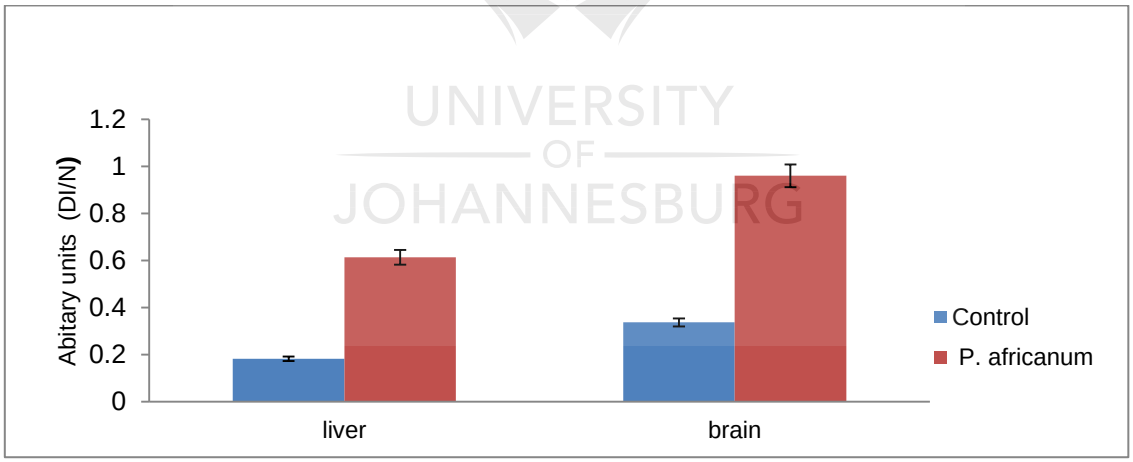


Figure 4.14: The levels of Ascorbyl radicals produced in the liver and brain homogenates of mice administered with samples of *P. africanum* extract

4.3.9.2 ROS (PBN) levels in organ homogenates of mice

The ROS levels (Figure 4.15) generated when mice were treated with *P. africanum* extract were generally higher than those of the untreated mice. In the liver, ROS

mean level of 1.707 was recorded, which was higher than the 0.66699 mean levels of ROS in the control. In the brain homogenates, ROS mean level of 1.7235 was recorded when mice were treated with *P. africanum* extract, which was more than 5 times higher than that 0.3167 obtained in untreated mice. These results are displayed in Figure 4.15 (kindly see also Table A3.8 of Appendix III).

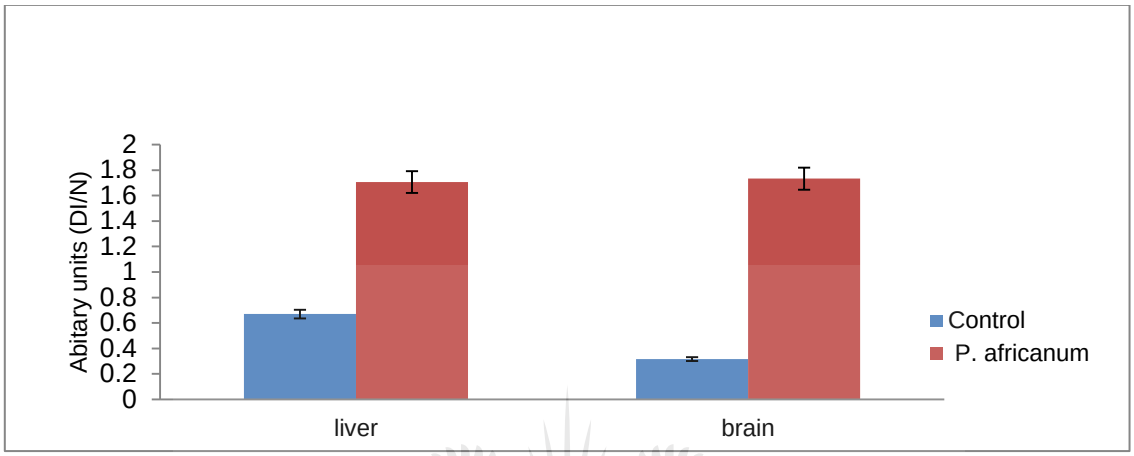
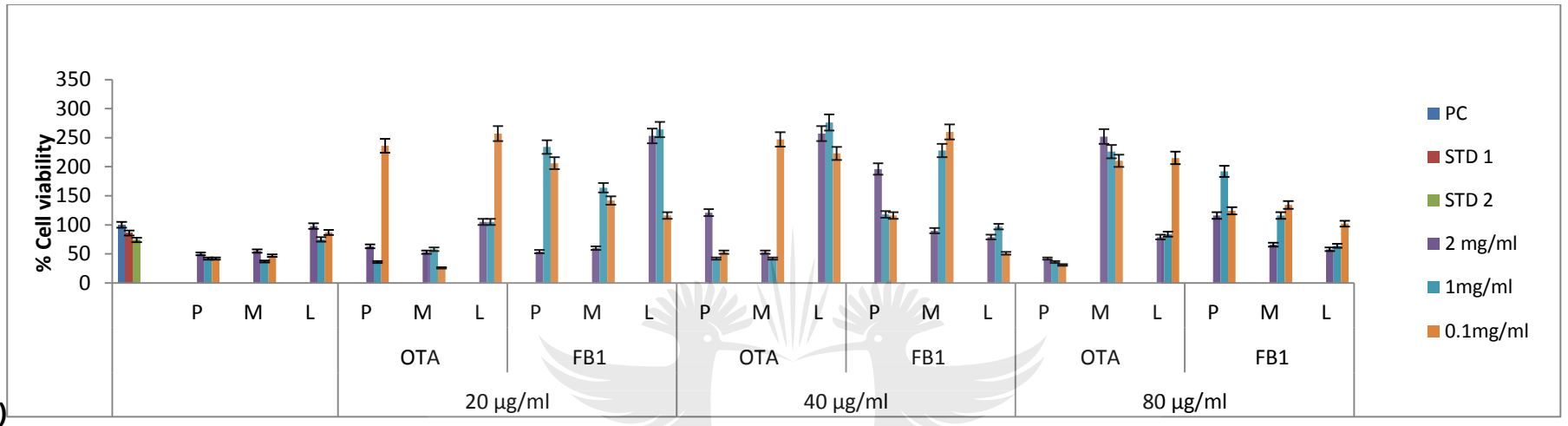


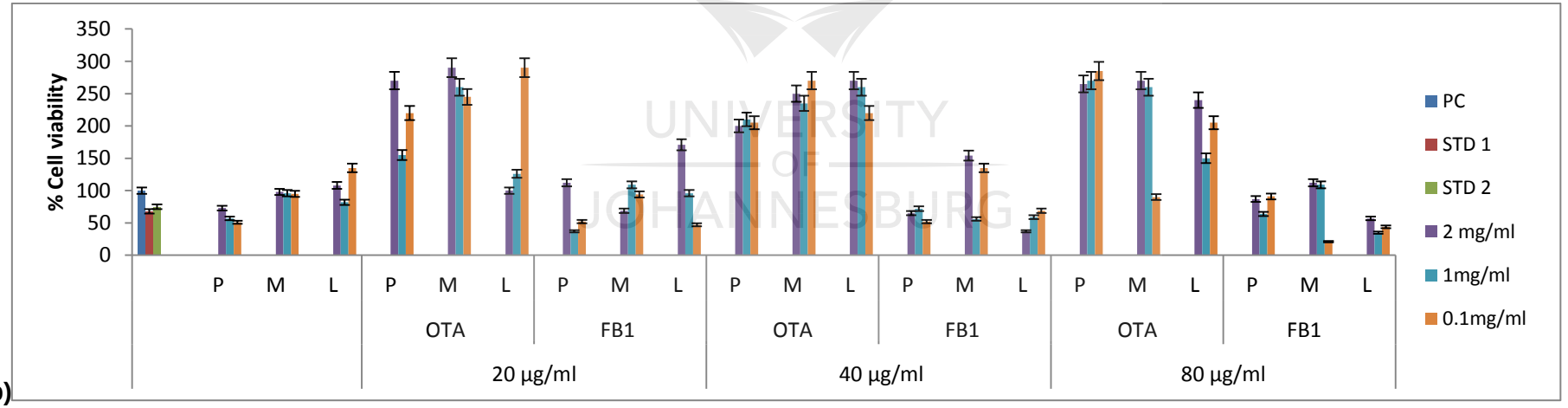
Figure 4.15: The levels of ROS/PBN radicals (expressed in arbitrary units) in organ homogenates of untreated (control) and treated mice with *P. africanum* extract

4.3.10 The cytotoxicity studies using methyl thiazol tetrazolium (MTT) assay

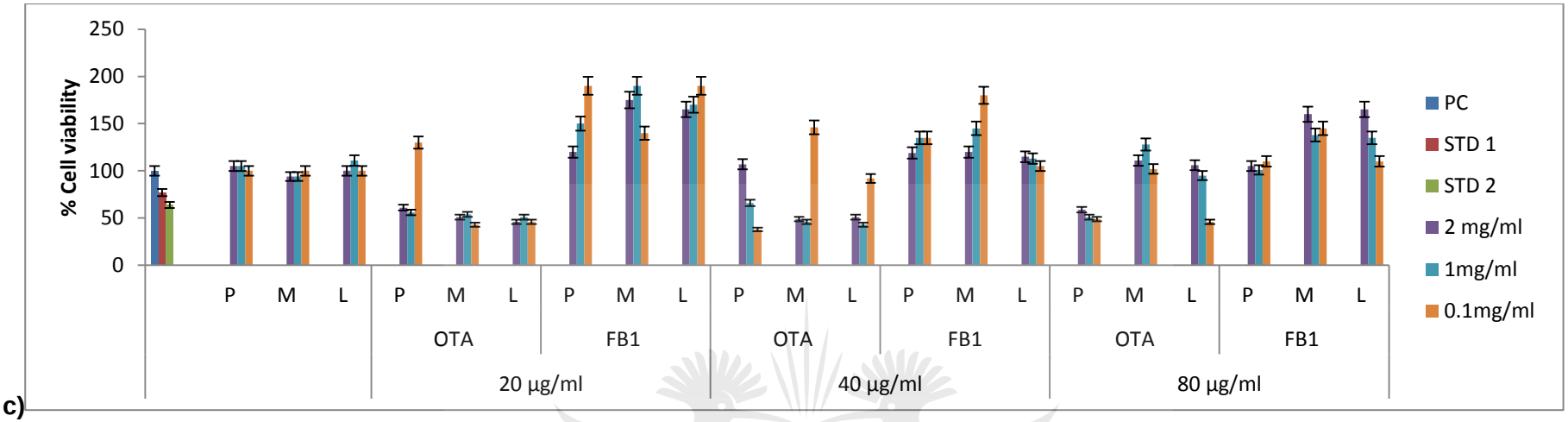
The cytotoxicity studies were conducted following the MTT assay to evaluate the effect of exposure of human lymphocyte cells to the three extracts of *M. longifolia*, *L. leonurus*, and *P. africanum*. Generally results revealed some cytotoxic effects observed at high concentration (2 mg/ml) than at low concentration (0.1 mg/ml). In addition, the MTT assay evaluated the cytoprotective activity of the three plant extracts on lymphocytes exposed to the toxins OTA and FB_1 for 24, 48 and 72 hrs. Generally, while exposing the cells to FB_1 resulted in an increase in cell proliferation over time of exposure, % cell viability decreased when OTA was added to the cells irrespective of the plant extract and level of toxin added (Figure 4.16).



a)



b)



c)

Figure 4.16: Percentage viability of human lymphocytes exposed to concentrations of *M. longifolia* (M), *L. leonurus* (L), and *P. africanum* (P) extracts and mycotoxins (OTA and FB_1) after a) 24 hrs b) 48 hrs and c) 72 hrs intervals. The positive control is denoted (PC), mycotoxin standards (STD 1: FB_1 and STD 2: OTA) and the concentration of plant extract at 2-0.1 mg/ml.

CHAPTER FIVE

5. DISCUSSIONS

5.1 Extraction efficacy

Solvent choice is largely dependent on the purpose of extraction (Das *et al*., 2010; Tiwari *et al*., 2011) and that extraction efficiency depends on the polarity of the compounds and that of the solvent (Bunghez *et al*., 2013). In this study, extraction of bioactive components from herbal plants was of interest, though it was not obvious which solvent would extract the active fractions the most. Thus, different polarity indexed solvents were used for extraction which increased the extractability of active components. Hexane, the least polar solvent used was the least effective irrespective of the plant material. Highest yields of 8, 7.3 and 6.6 % were notable obtained in methanol extracts of *M. longifolia, L. leonurus,* and *P. africanum*, respectively. This is in line with the report of Tiwari *et al*. (2011). It can thus be concluded that more polar than nonpolar compounds were present in these plant materials under study since each solvent can only extract those components that are readily soluble in it.

5.2 Phytochemical analysis

5.2.1 Chromatographic profiles

The choice of solvent system for developing TLC for effective separation of plant components is important. A variety of solvent systems were tested. The developing solvent consisted of Benzene: ethanol: ammonium hydroxide 18:2:02 (BEA) as developed by Eloff *et al*. (2005) was the best solvent system that efficiently partitioned and separated the plant components into several bands when compared to chloroform: ethyl acetate: formic acid 10:8:2 (CEF) or ethyl acetate: methanol: water 10:1.35:1 (EMW) (Figure 4.2). The use of visualization techniques enhances the quantifiability of the separated compounds. The most distinct bands after spraying with sulphuric acid and their R_f values recorded were 9, 14, and 10 for *M. longifolia*, *L. leonurus*, and *P.africanum*, respectively. This indicates that *L. leonurus* had the highest number of compounds separated by TLC. The following extracting solvents recorded the highest separated compounds in the samples: DCM and EtAc in *M. longifolia* (9 R_fs), EtAc in *L. leonurus* (13 R_fs) and EtAc in *P. africanum* (8 R_fs).

The presence or absence of bands amongst the extracts can only be a question of the ability of a specific solvent to solubilize a compound from the plant material. Generally, it is expected that extraction using various solvents would enable optimum extraction of those compounds that are soluble in a particular solvent. Additionally, traces of some co-eluting compounds can easily be separated and concentrated with the next extraction solvent, especially when sequential extraction is conducted, as observed in this study. As such, there were bands of compounds that had close approximate R_f values with other compounds. For instance, the R_f values 0.17 and 0.18 of *L. leonurus* in Table A1.2, which were in lanes 1, 2 and 3 (Hex, DCM, and EtAc) in the chromatogram, are in a close proximity. It is either the solvent system could not distinctively separate them or they have very close masses and therefore co-eluted. Although, each band may seemingly look like one compound, it can contain several other compounds (grouped together according to their affinity with the solid phase) that require further purification in order to distinctively obtain a pure band compound.

5.2.2 UV-Vis spectroscopy

The UV-Vis fingerprint results presented in Table 4.3 range within the typical characteristic absorption peaks of phytochemicals found in herbal extracts. From the UV-Vis spectra, we were able to determine different classes of polyphenolic compounds present in these plants. For example, the presence of phenolic compounds like flavonoids which absorb UV-Vis in the range of 230-290 nm (Pinheiro and Justino, 2012; Deepa *et al*., 2014) seen in *M. longifolia* at peak 245 nm. Polyphenols range between 280-330 nm, represented in *M. longifolia*, *L. leonurus* and *P. africanum* at peaks 294, 295, 300 and 305 nm, respectively. Phenolic acids and their derivatives such as flavols, flavones, quinones and phenylpropenes have characteristic absorption peaks in the 330-420 nm region, while quinones and chlorophylls are found between 400-412 nm (Bunghez *et al*., 2013) represented by peak 412 nm in *L. leonurus*. The presence of phenolic compounds determined by UV-Vis were further confirmed by the presence of their functional group bond frequency vibrations using FT-IR.

5.2.3 FT-IR spectroscopy

In this study, the mass spectra of absorbance peaks obtained by FT-IR spectroscopy from extracts of *M. longifolia*, *L. leonurus*, and *P. africanum* extracts (Figure 4.3), were mostly typical functional groups found in phytochemicals as reported in other studies (Johnson *et al*., 2012; Raman *et al*., 2013). Unique to *L. leonurus* were some aldehydes and ketones presenting at 1732.47 cm^{-1}, while nitro compounds and cyclohexane ring vibrations were uniquely present at 1506.80 and 1031.34 cm^{-1} in the *P. africanum* extract. The most common functional groups in the three extracts included alcohols, phenols, alkenes, ethers, alkynes, carboxylate, alkanes, secondary amines, esters, and carboxylic acids. These are the basic functional groups of the compounds identified when we applying 2D GCxGC-TOF/MS. For example, the phenol/alcohol functional groups (Table 4.2) with bond/group frequencies of 3411.66, 3411.5, and 3314.92 cm^{-1} respectively, correspond to the presence of monoterpenes confirmed within the plant extracts. These include such monoterpenes identified from *M. longifolia* as l-menthone, camphene, and à-terpineol. Those from *L. leonurus* included cinnamaldehyde, (E) - 2-caren-10-al and 2H-pyran-2, 6(3H)-dione. The 2D GCxGC-TOF/MS confirming the presence of these phytochemicals in the test plant materials are discussed subsequently.

5.2.4 2D GCxGC-TOF/MS

Numerous compounds were identified by 2D GCxGC-TOF/MS within each plant extract. The focus then became only on those that had 70% similarity and above, since they depict similar skeletal forms with the known compounds (NIST and Adams EO library) and some differences due to the presence or absence of unique side chains as well as the occurrence of isomers. Out of these, 6 compounds per plant extract were selected based on some of their phytochemical properties (Tables A1.3, A1.4, and A1.5 in Appendix I) displayed in Figures 4.4, 4.5, and 4.6. These properties (antioxidant, anti-inflammatory, anticancer, antimicrobial, antifungal etc.) are key phytochemical elements sought after for possible inclusion as animal feed components to limit exposure or reduce the toxic effects of mycotoxins. For example, the flavonoid quercetin reported to have antioxidant and anti-inflammatory activity, also effectively functions as a cytoprotection agent against ochratoxin A (OTA) induced toxicity in human lymphocyte cells (Periasamy *et al*., 2014). Lupeol, a

pentacyclic triterpenoid with anti-inflammatory, anticarcinogenic and antimicrobial activity has also been reviewed to have hepatoprotective action against aflatoxin B_1 (AFB_1) induced toxicity (Sigh *et al*., 2014).

Mass spectroscopy gives characteristic fragment ions which enable the identification of different classes of phytochemicals (Ekanayaka *et al*., 2015). The mass of the generated ions were measured with time-of-flight (TOF) analyzer which detects ion velocity reported as mass-to-charge ratio (*mz*) (Pinheiro and Justino, 2012). The fragmentation during analysis depends on the structural substitution which differs with unique phytochemical classes (Cuyckens and Claeys, 2004).

The 6 compounds and their mass spectra from *M. longifolia* (Figure 4.4) were

1) á-pinene, a terpene with antioxidant and antimicrobial activity (Silvério *et al*., 2013), had a spectrum at retention time 320, peak no. 41, relative content at 0.76%, had a major base ion at (*mz*) 93;

2) I-menthone, a monoterpene used as a derivative for menthol (Kamatou *et al*., 2013), produced strong molecular fragment ions at *mz* 41/112, at a relative content of 0.45%;

3) apocynin had a quantifier fragment ion with *mz* 151 and two quantitator fragments at *mz* 65/103, present at 0.05%, a monoterpene with anti-inflammatory and antioxidant activity (van den Worm *et al*., 2001).;

4) naphthalene, 1, 2, 3, 5, 6, 8a, hexahydro-4, 7-dimethyl-1(1-methylethyl)-(1S-cis)-, had a base ion with *mz* 119/105, present at 0.01%, a sesquiterpene with antifungal activity (Wang *et al*., 2011);

5) camphene that gave a major fragment ion at *mz* 93, present at 0.02%, is described as a terpenoid with antioxidant and cytoprotective activity (Tiwari and Kakkar, 2009); and

6) à-terpineol, released a major fragment at *mz* 59, present at 0.13%, a monoterpene alcohol with anticancer activity (Hassan *et al*., 2010).

Despite the significance of these properties, few if any, have been applied as antimycotoxin therapeutic agents, especially as feed additives. The challenge would be in determing whether the compounds work best by having some synergistic or additive effects at experimental doses.

Those 6 compounds selected in methanol leaf extract of *L. leonurus* (Figure 4.5) were:

1) 1-methyl-pyrrolidine-2-carboxylic acid that gave major fragment with *mz* 84, present at 8.66%, a carboxylic acid with antimycotoxin activity, reported to interfere with aflatoxin (AF) synthesis at transcriptional level (Murugan *et al.*, 2013);

2) 5-hydroxypipecolic acid, which produced a major fragment at *mz* 82, present at 1.66%, is described as an imino acid used for the synthesis of anticancer drug XV710 (Vranova *et al*., 2013);

3) cinnamaldehyde, (E)-, with a quantifier fragment ion *mz* 131 and two quantitator fragments with *mz* 51/77, present at 3%, is described as a monoterpene with antioxidant, anti-inflammatory and anti-cancer activity (Rao and Gan, 2014);

4) 2, 4-dihydroxy-2,5-dimethyl-3(2H)-furan-3-one, that which gave a fragment ion with *mz* 101, present at 0.25%, and described as a furanone with antioxidant activity (Schwab, 2013);

5) 2-caren-10-al, gave a strong molecular fragment ion peak at *mz* 77 with quantitator fragments at 65/107, relative content at 0.08%, is a monoterpene with antioxidant and antimicrobial activity (Hajlaoui *et al*., 2010); and

6) 2H-pyran-2, 6 (3H)-dione, gave a characteristic fragment at *mz* 55, present at 0.15% a monoterpene with anticancer activity (Kranjc and Kocevar, 2013).

From the methanol leaf extract of *P. africanum*, 6 compounds were selected (Figure 4.6) they included:

1) resorcinol *mz* 110, present at 30.1%, described as a phenol used for the manufacture as an antixidant food additive (EFSA, 2010b);

2) N,N-dimethylglycine, with *mz* 55, present at 4.25%, an amino acid glycine which has anti-tumor, antibacterial, anti-inflammatory activity and is used as an anti-stress nutrient supplement (Natural Medicines Comprehensive Database, 2015);

3) indole which fragmented and gave a major ion fragment at *mz* 117, is described by Biswal *et al*. (2012) as an aromatic compound with antimicrobial

activity used as the main component in the manufacture of anticancer and antidepressant drugs;

4) phenol, 2, 6-dimethoxy-4-(2-propenyl)-, with major fragment peak at *mz* 81, occurred at 0.03%, a phenylpropanoid used as a flavourant (Burdock, 2001);

5) vanillin, a methoxyphenol with a characteristic a major base ion fragment at *mz* 151, present at 0.07% has anti-inflammatory activity (Murakami *et al.*, 2007); and

6) pantolactone with *mz* 71, present at 0.12% described as a lactone used as a feed additive (Hilterhaus and Liese, 2007).

It was also observed that there were 12 predominant compounds that were present in 2 or 3 of the plant extracts tested as seen in Figure 4.7:1-12 have been reported to have various pharmaceutical properties especially those considered relevant to this study. The constituents were

1) phenol 2,6-dimethoxy fragmented to a base at *mz* 154;

2) phytol with the major fragment at *mz* 71;

3) n-hexadecanoic acid which gave a characteristic fragment with major ion;

4) 2-methoxy-4-vinylphenol at *mz* 135;

5) 2,2'-bioxirane with a base fragment ion *mz* 55;

6) 4H-pyran-4-one 2, 3-dihydro-3, 5-dihydroxy-6-methyl- a quantifier fragment ion at *mz* 43 quantitor fragments *mz* 55/101;

7) 2-propanone, 1-hydroxy- with 2 major fragment ions at *mz* 74/43;

8) 3,7,11,15-tetramethyl-2-hexadecen-1-ol with *mz* 41;

9) furfural which gave major fragment at *mz* 39;

10) pulegone gave major fragment at *mz* 67;

11) 1,2-cyclopentanedione fragment ion at *mz* 55 and quantitor fragment ions at *mz* 42/98; and

12) á-D-glucopyranose, 1,6-anhydro- with a major fragment ion at *mz* 60 and quantitator fragment ions at *mz* 42/73.

Although, these compounds co-occurred in these plant extracts, their concentrations differed, which could be used to consider which of the plant could potentially be used to source these compounds i.e. using plant biotechnological application like tissue culture. For example, the diterpene phytol which has anticancer, antidiabetic, anti-inflammatory, antioxidant and antimicrobial activity (Pejin *et al*., 2014) occurred at

0.36% in *M. longifolia* compared to 1.16% in *L. leonurus*. The palmitic acid, n-hexadeconoic with antioxidant activity (Prabu *et al*., 2013) occurred at 0.35% in *M. longifolia*, and in *L. leonurus* at 0.63%. The flavonoid 1, 2, 3 4 H-pyran-4-one, 2, 3-dihydro-3, 5-dihydroxy-6-methyl-, with antioxidant and anti-inflammatory activity (Yu *et al*., 2013) was present at the 0.2% in *M. longifolia*, 0.02% in *L. leonurus*, and at 0.07% in *P. africanum*. The terpene alcohol 3, 7, 11, 15-tetramethyl-2-hexadecen-1-ol, with antimicrobial and anti-inflammatory activity (Kalaimagal and Umamaheswari, 2015) occurred at 0.13% in *M. longifolia* and at 0.19% in *L. leonurus*.

The assessment of % yield of each compound evaluates the potential nutritional, cytotoxic and therapeutic value of the constituents present, taking into consideration that the study looks at the possibility of making use of each of the 3 plants as animal feed additives. These additives would even be more desirable if they also provide the extra nutrients or minerals alongside the therapeutic element. This concept is not new as it has been applied with the famous *Moringa oleifera* leaves which are relatively rich in protein and are being added to animal feed with positive fattening results (Melo *et al*., 2013). The top 5 abundant compounds in *M. longifolia* in Table 4.3 include 2-propanone, 1-hydroxy- at 33.6%, sucrose 13.05%, acetic acid at 8.4%, acetic anhydride at 7.05% and formic acid at 3.04%. The top 5 in *L. leonurus* in Table 4.4 were acetic acid at 13%, acetic anhydride at 10.1%, sucrose 8.99%, 1-methyl-pyrrolidine-2-carboxylic acid at 8.66% and 2, 2'-bioxirane at 5.34%. The top 5 most abundantly occurring compounds in *P. africanum* in Table 4.5 were resorcinol at 30.4%, acetic acid at 16.8%, phenol, 2-methoxy- at 4.65, N,N-dimethylglycine 4.25%, and carbamic acid and monoammonium salt at 3.13%.

The phytochemical screening of *M. longifolia* extracts are shown to contain alkaloids, flavonoids, cardiac glycosides, phenolics, tannins, monoterpene ketones, saponins and terpenes (Ghoulami *et al*., 2001; DoNascimento *et al*., 2009; Ashfaq *et al*., 2012). The major phenolic constituents are caffeic acid derivatives (Tekeľova *et al*., 2009) and polar flavonoids (Ghoulami *et al*., 2001; Akroum *et al*., 2009; Tekeľova *et al*., 2009) as well as quercetin and kaempferol glycosylated derivatives, glycosylated flavonoids (Al-Bayati, 2009). The presence of polyphenols, flavonoids and condensed tannins has been shown to correlate with the antioxidant activities in aerial parts of this plant (Hajlaoui *et al*., 2009; van Wyk *et al*., 2009).

The main phytochemical compounds isolated from *L. leonurus* include tannins, alkaloids, saponins, terpenoids, quinones, triterpenes and steroids as reviewed by Ascensao and Pais (1998). Phytochemical studies on extracts of *P. africanum* have showed the presence of most of these compounds in addition to flavones, saponosides and glycosides (Mengome *et al.,* 2009; Akinlami *et al*., 2012) as well as carbohydrates, sterols, glycosides (Akinlami *et al*., 2012) and triterpene glycosides (Noté *et al*., 2013). Mengome *et al*. (2009) isolated sapanosides from ethanolic extract of *P. africanum*. Brusotti *et al*. (2013) isolated saponin fraction with antifungal activity while the expressed radical scavenging activity in this study can be linked to the presence of constituents such as tannins and flavonoids in this plant (Grigorov *et al.*, 2014).

5.3 Biological assays

5.3.1 Agar diffusion assay

Based on the activity of the plants to inhibit fungal growth, we found that antifungal activity of the methanol extract of the South African *M. longifolia* (cultivated) against *A. niger and A. flavus* was insignificant. This finding agrees with the results obtained by Hajlaoui *et al*. (2009) but contradict with the moderate activity reported on Iranian methanol extracts (Razavi *et al*., 2012). This may suggest that cultivated plants have a much less activity than wild growing plants as well as emphasizes on the importance of an assessment of the factors like geographical location that influence the variability in quantity and quality of bioactive components in plants (Al-Ankari *et al*., 2004), when harvesting plant material for biological assays.

The most prominent lasting activity of *P. africanum* extract was recorded against *A. flavus*, *A. parasiticus*, and *A. niger* up to the 7th day. The extract showed a higher activity (MIZ of 25 mm) against *A. niger* on Day 3 when compared to the control (Amphotericin B (AmB)) with an MIZ of 15 mm as seen in Table 4.6. *Aspergillus fumigatus* showed resistance to both the plant extract *P. africanum* and AmB. The highest % minimum inhibition zone (%MIZ) was also observed on day 3, as 1.56 % against *A. niger*. This therefore suggest that the extract in pure form can be used as a mold inhibitor in animal feeds to prevent growth of *A. flavus*, *A. parasiticus*, and further limit aflatoxin production by these fungi, though this should be studied further

in vivo. Additionally, the same extract can equally be used in formulating drugs in the treatment of diseases caused by these fungi in animal and man, however, the fungicidal activity of the extract should be studied further to ascertain this view.

The presence of a compound that has 80% similarity to the fungicidal compound furfural at peak 27, relative content of 0.31% in the methanol extract of *P. africanum*, may be linked to the observed antifungal activity. Some of the compounds identified by 2D GCxGC-TOF/MS in these plant extracts were labeled unknown because they are not listed in the databases of the hint library softwares used (NIST, Adams EO library), yet these could be some of the compounds that could account for the observed antifungal activity in the *P. africanum* extract reported in this study.

5.3.2 Microplate dilution assay

The 5 fungal species tested against *P. africanum* are common and important disease-causing as well as mycotoxin producing fungi. The lowest concentration with growth inhibition recorded in Table 4.7 for *A. flavus* and *A. parasiticus* was at 2 mg/ml of *P. africanum* extract. While the species *A. fumigatus*, A. *ochraceus* and *A. niger* were each inhibited by 4 mg/ml of the extract. The expected results were unimpressive considering antifungal activity studies of some plant extracts by Adamu *et al*. (2012) and Mamba *et al*. (2010) who found MIC values as low as 0.02 mg/ml. In addition, an MIC of 0.03 mg/ml of a *P. africanum* saponin fraction against *Pyricularia grisea,* a rice blast causing fungi was reported by Brusotti *et al*. (2013). Obviously, the crude extract used in this study did not have a high MIC value when compared to this pure fraction. Also, the *Pyricularia grisea* could be more sensitive to *P. africanum* and the tested mycotoxigenic *Aspergillus* species were rather more resistant. Similar results were reported by Mahlo *et al*. (2010) where the tested plant extracts had 0.02 mg/ml MICs against *Penicilliun expansum* and *P. janthinellum,* while *A. niger and A. parasiticus* were more resistant even at minimum inhibitory doses above 1 mg/ml.

5.4 EPR spectroscopy antioxidant activity studies

5.4.1 Free radical activity

The EPR spectra recorded for all 3 plant extracts in powdered sample before and after 2 hrs of UV irradiation exhibited a singlet spectral line seen in Figures 4.8a and b., were similar to data reported in Karamalakova *et al*. (2013). The intensity of the spectrum is said to be directly proportional to the concentration of free radicals in the sample (Ramos *et al*., 2013). Treatment of antioxidant components with UV irradiation also tests the stability of radical structures, since oxidation generates unstable radicals in some natural antioxidants with phenolic groups (Adhikari *et al*., 2012). This is because under UV irradiation, chemical bonds of the antioxidative components may be broken and the distance between unpaired electrons may be altered (Ramos and Pilawa, 2015). The antioxidant components present in the plant extracts tested in this study were rather stable when exposed to UV irradiation (Figure 4.8b). The intensities of EPR signals registered in powdered form were not affected by UV irradiation ($p>0.05$), similar to the data obtained by Adhikari *et al*. (2012). Therefore, decontamination with UV irradiation is the suggested suitable processing procedure for antioxidants (Fatemi *et al*., 2014) including the proposed feed additives that would be achievable without drastic changes in their function as effective antioxidants

The paramagnetic resonances represented by g-values recorded in each extract fall under the normal expectation for the presence of a free electron, which is between 2.00232 and 2.00000 ± 0.0002 for organic radicals (Suhaj *et al*., 2006; Osorio *et al*., 2011; Karamalakova *et al*., 2013). The g-value is determined from the value of the magnetic field at the middle of the spectrum which arises from the free electron spin-orbit coupling between the ground and excited states (Duin, n.d). The highest g-value recorded before 2 hrs UV irradiation is 2.00993 for the extract of *L. leonurus,* while 2.01123 is the highest g-value recorded after 2 hrs UV irradiation for both *M. longifolia* and *P. africanum*. Interestingly, these were recorded as new radicals that formed after exposure to UV irradiation. The treatment with UV also evaluates the decay of present radicals as well as the formation of new radicals as seen in Figure 4.8b. Therefore, irradiation as discussed in Pérez *et al*. (2007) and Polovka and Suhaj (2010) produces free radicals which oxidize phytochemicals breaking some of

the chemical bonds into more soluble phenols, thus the formation of new radicals and elevated antioxidant activity.

5.4.2 The % DPPH scavenging activity

The spectra of each of the 3 extracts in Figures 4.9 b), 4.10 b), and 4.11 b) are similar (single line spectra) because they are all dissolved in the same solvent, ethanol. In solution, orbitals of the species with radical activity are oriented in the molecule depending on the direction (anisotropic) such that in solution, anisotropy is averaged out, thus the similarity in the spectral line (Duin, n.d).

In general, the % DPPH activity parameters of the EPR spectra depend on the concentration of free radicals provided by DPPH in ethanol solution. Thus, the measurement of the % DPPH scavenging activity of each plant extract essentially reflects how much of the DPPH free radicals can be scavenged by the antioxidant species present in each extract (Sowndhararajan and Kang, 2013). That is why the DPPH concentration is kept constant while the concentration of the species in question can be varied.

All the three plant extracts displayed a concentration dependent % DPPH scavenging activity (Tables A3.1, A3.2, and A3.3; Figures 4.9a , 4.10a, and 4.11a) and a comparable trend was previously obtained by Raj *et al*. (2010) and Adhikari *et al*. (2012), although there is a drastic difference in each plant extract. For example, at 0.1% stock concentration using 30 µl, *M. longifolia* had a 40% DPPH scavenging activity, while *L. leonurus* recorded a higher activity of 69% under the same conditions. *P. africanum* recorded a much higher antioxidant activity of 99% under the same concentration but a lower volume of 10 µl. It was obvious that *P. africanum* had superior antioxidative activity followed by *L. leonurus* and the least activity exhibited by *M. longifolia* in this study. It was also noteworthy that *P. africanum* had a higher antioxidant activity when compared to the native Bulgarian plant *Haberlea rhodopensis* under the same experimental conditions (Grigorov *et al.,* 2014). The DPPH scavenging activity of *L. leonurus* found herein agrees with the good antioxidant activity results by Odeyemi and Afolayan (2010) and Jimoh *et al*. (2010).

Strong or weak antioxidant activity is largely dependent on the phytochemical composition (Santos *et al.,* 2009; Karamalakova *et al*., 2010; Ghosh *et al*., 2013; Mohammed *et al*., 2013) of a plant extract. Examples include the presence of tannins and flavonoids in the *P. africanum* extract (Grigorov *et al*., 2014) as well as saponins (Brusotti *et al*., 2013) and hydroxycinnamic derivatives and flavonoids in *M. longifolia* (Tekeľova *et al*., 2009).

The antioxidant capacity of *M. longifolia* extracts varies when different extraction methods and antioxidant assays, i.e., DPPH or 2,2'-azinobis-3-ethylbenzothiazoline-6-sulfonicacid (ABTS) or ferric decreasing antioxidant property (FRAP) assay are used (Mikaili *et al*., 2013). *Mentha longifolia* was reported to be a better scavenger of ABTS when compared to DPPH and FRAP (Raj *et al*., 2010), possibly due to the differences in the electron reduction potential of each assay (Nickavar *et al*., 2008). The low antioxidant activity exhibited by *M. longifolia* methanol extract using DPPH was observed in this study possibly, higher activity if the ABTS assay was conducted could have provided better results, although, this is largely dependent on the total phenolic composition of the extract (Nickavar *et al*., 2008). The low DPPH scavenging capacity of *M. longifolia* reported in this study is comparable to results presented by Nickavar *et al*. (2008) and Al-Ali *et al*. (2013). Despite that, methanolic extract have been shown to exhibit a higher activity than the essential oil fractions (Hajlaoui *et al*., 2009; Ertaş *et al*., 2015).

The 2D GCxGC-TOF/MS results confirmed the presence of á-pinene, apocynin, camphene, cinnamaldehyde, (E)-, 2,4-dihydroxy-2,5-dimethyl-3(2H)-furan-3-one and N,N-dimethylglycine, phytol, n-hexadecanoic acid, and 4H-pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-,compounds that may be responsible for the antioxidant activity exhibited by the three plant extracts as earlier discussed in Section 5.2.4.

5.4.3 The effects of 2 hrs UV irradiation and high ultrasonic waves on % DPPH scavenging activity of *P. africanum* extract

Based on that data generated and reported herein, it was observed that 2 hrs UV irradiation and exposure to high ultrasonic waves of *P. africanum* extracts gave inconsistent results such that at 10 µl, %DPPH increased from 45.06 to 54.53% whereas a slight decrease from 98.17 to 86.89% was found when 50 µl of the extract

was applied. This can be compared to the % DPPH scavenging activity before UV irradiation and exposure to high ultrasonic waves in Figure 4.12. This is due the fact that the volume and concentration of *P. africanum* samples and the concentration of DPPH were similar. Meanwhile, an assessment of each assay singularly still showed the concentration dependent increase in % DPPH scavenging activity similar to what was observed by Adhikari *et al*. (2012). The increase in % DPPH scavenging activity of *P. africanum* after exposure to UV irradiation (Figure 4.12) was similar to data reported by Wei *et al*. (2013) in *Cajanus cajan* leave extracts which linked UV irradiation with increased levels of phenolic content and antioxidant activity.

5.4.4 The effects of 10, 30 min and 24 hrs incubation on % DPPH scavenging activity of P. africanum extract

The % DPPH scavenged at low (0.1%/10 µl) concentration displayed in Table A3.6 and Figure 4.13 increased with time from 8.51% after 2 hrs UV irradiation to 43.51% after 24 hrs incubation, which is a difference of 35%. Increasing the concentration to 0.1%/50 µl had an insignificant effect, although a difference of 3.5% was seen. Similar increases in DPPH scavenging activity of *Justicia adhatoda* methanol extracts were reported in Rajurkar *et al*.(2012) after exposure to gamma irradiation. The increase was linked with the formation of Maillard reaction products (MRPs) which effectively scavenge radicals thus the enhanced antioxidant activity. Furthermore, high levels of flavonoid were linked with enhanced antioxidant activity after irradiation of *Zizyphus mauritiana* fruit extract (Kavitha *et al*., 2015). In contrast, Heo *et al*. (2015) reported a drastic decrease in the DPPH scavenging activity of the flavonol quercetin after UV irradiation due to destruction of the B-ring and A-C benzoyl structures. Thus, the formation or degradation of radicals depends on the stability of the radical structural intermidiates.

As is seen in Table A3.5 and Figure 4.13a, differences found between the 10 min and a 30 min incubation interval is significant. At 10 min interval, there is an increase of 16% DPPH scavenged while at the 30 min interval an increase of 59% DPPH scavenged after 2 hrs UV irradiation was seen. The results obtained agree that the sample interaction with DPPH is depended on the time of exposure to UV irradiation and that EPR spectroscopy sensitively detects specific short or long-lived radicals (Spasojević *et al*., 2011).

In addition, the effect of incubation time on the antioxidant activity after UV irradiation is an assessment of the effect of radiation preservation treatments on prospective phenolic antioxidants. Elevated antioxidant activity not immediately after UV irradiation but rather after incubation intervals were reported by Suhaj and Horváthová (2007) similar to those reported in this study Figure 13b. Therefore, the storage time after irradiation does have an effect on the antioxidant activity (Kavitha *et al*., 2015) although it is most likely to decrease over longer periods of storage (Suhaj and Horváthová, 2007).

5.4.5 Superoxide dismutase (SOD) activity

The *P. africanum* extract only displayed SOD-like activity before exposure to UV irradiation and no SOD-like activity was recorded after 2 hrs UV irradiation and at 24 hrs after incubation. This assay was carried out to determine the capacity of antioxidant species in question to scavenge ROS, specifically the superoxide anion (Jia *et al*., 2008) by the action of antioxidant enzymes such as SODs (Pejić *et al*., 2008). This thus, predicted the SOD-like activity of the plant extract (Hou *et al*., 2013). The results indicated that *P. africanum* has 12.22 U/mg protein compared to the control with a value of 6.04 U/mg protein SOD activity by Zheleva *et al*. (2005). Thus, this suggests that *P. africanum* expressed antioxidant activity both *in vitro* and *in vivo* and it therefore, is desirable as a potential candidate in reducing oxidative stress-induced mycotoxin toxicity in animals. In addition, a decrease in SOD-like activity below the normal levels means an increase in oxidative stress, such that the antioxidant and hepatoprotective activity of a plant can be measured with the SOD-like activity (Padmalochana *et al*., 2013).

5.4.6 *Ex-vivo* studies

5.4.6.1 Ascorbyl (•Asc) radical level production in tissue homogenates

The measure of ascorbly (•Asc) radicals is also one of the best endogenous biomarker assays in determining the oxidative status of a biosystem, because ascorbate antioxidants present are easily detected by EPR (Spasojević *et al*., 2011). The result indicates higher values than the control treated with solvent only after the exposure of mice to *P. africanum* extract. In addition, higher levels of •Asc were seen

in the brain samples than on the liver samples. Thus, *P. africanum* extract according to the results in Table A3.7 and Figure 4.14 acts as a pro-oxidant, which is the case with some phenolic antioxidants (Karamalakova *et al*., 2010; Yordi *et al*., 2012). These results contradict the high antioxidant activity recorded by this extract, possibly implying that *P. africanum* has some toxic components despite its antioxidant activity. Furthermore, the high levels of •Asc radicals after exposure to *P. africanum* suggest that this plant does not have hepatoprotective effects like the case seen in artichoke *Cynara scolymus* Linn with lower •Asc radicals (Georgieva *et al*., 2012). One would propose extract enrichment through the isolation and elimination of any toxic components (like cardiac glycosides) present. This however, was not considered in the present study but further testing the *in vitro* and *in vivo* antioxidant activity of enriched isolates, would be ideal to pursue.

5.4.6.2 ROS (PNB) radicals level production in tissue homogenates

The reactive oxygen species (ROS) represented as n-tert-butyl-alphaphenylnitrone (PBN) radicals produced both in the liver and brain of mice after treatment with *P. africanum* extracts were indicative of the production of high ROS levels in these organs as displayed in Table A3.8 and Figure 4.15. This suggest that *P. africanum* is also acting here as a pro-oxidant producing radicals instead of scavenging them. It is logical that *P. africanum* extract could act as pro-oxidants due to the presence of flavornoids, which are reported to sometimes exert pro-oxidant instead of exhibiting some antioxidant properties (Karamalakova *et al*., 2010).

The •Asc and ROS products (presented as PBN rad.) determined by EPR spectroscopy are real-time biomarkers for the oxidative status in the livers and brains of the tested healthy mice (Buettner and Jurkiewicz, 1993; Spasojević *et al*., 2011; Zheleva, 2012). A comparison of the results obtained (Tables A3.7 and A3.8) indicates that the levels of •Asc and ROS products in the organs of controlled mice versus those dosed with *P. africanum* extract, indicate that *P. africanum* is toxic. Obviously, *P. africanum* extract provokes an even higher degree of oxidative stress. These results are contradictive to the *in vitro* studies which showed potential antioxidant activity of *P. africanum* expressed by the 99% DPPH scavenging and SOD-like activity. Again, this emphasizes the importance of conducting both *in vitro*

and *in vivo* studies to confirm the integrity of a potential extract for inclusion in feed in real situations in the field.

5.5 Cytotoxicity studies

It is generally expected that exposure of animal tissues to mycotoxins results in apoptotic and necrotic effects (Makun *et al*., 2011; Al-Hammadi *et al*., 2014; Mwanza and Dutton, 2014), which ultimately result in decreased cell viability (Wan *et al*., 2013). In this study, the methyl thiazol tetrazolium (MTT) assay evaluated firstly, any possible cytotoxic activity exerted by the plant extracts alone on the lymphocyte cells *in vitro*. As seen in Figure 4.16a, OTA after 72 hrs of exposure with or without plant extract, exhibited cytotoxicity activity, whereas FB_1 exposure under the same experimental conditions resulted in increased cell proliferation. This thus demonstrates that OTA is more toxic when compared to FB_1. It has been found that FB_1 stimulated T cell proliferation in rats (Dombrink-Kurtzman *et al*., 2000), whereas human lymphocytes exposed to OTA caused a dramatic reduction in cell viability by 87% (Njobeh *et al*., 2009).

The extracts alone exhibited negligible cytotoxic effects as seen where % cell viability just below 100% at 24 hrs were noted. This may be explained by the fact that cells at 24 hrs exposure are still adapting to the environment and after 48 hrs later, there was cell proliferation, meanwhile after 72 hrs, they are in the lag phase. The least cytotoxic extract was *L. leonurus* compared to *M. longifolia* and *P. africanum*. A concentration dependent modest cytotoxicity of methanol extracts of *M. longifolia* were reported using MTT assay in McCoy cells (Razavi *et al*., 2012) as well as in MCF-7 cell line (Al-Ali *et al.*, 2013). Mikaili *et al*. (2013) reported that *M. longifolia* may exert some toxicity depending on the dose and thus not completely safe for its folkloric use in treating various ailments. Alcoholic root extracts of *P. africanum* have been reported to have antiproliferative activity against human colon cancer (CaCo-2) cell line (Mengome *et al*., 2009), thus the observed low % cell viability in both plant extracts. Furthermore, the observed pro-oxidant activity in the *in vivo* EPR studies as discussed in the previous (Section 5.4.5) suggests that *P. africanum* is cytotoxic.

It was expected that the varying concentrations (2, 1, and 0.1 mg/ml) would help determine at which concentration the extracts is more toxic or more cytoprotective,

as well as indicate which concentration can best be effective against which mycotoxin concentration, thus varying mycotoxin concentrations (20, 40, and 80 µg/ml) were tested. There were inconsistent patterns with the observed data as presented in Figure 4.16a, b and c. Therefore, it was difficult to establish which concentration of extract was effective. For example, the concentration of 0.1 mg/ml *P. africanum* at 24 hrs of exposure against 20 µg/ml OTA exhibited elevated % cell viability, which decreased with the increasing concentration of mycotoxin (40 and 80 µg/ml) tested for. This trend however, was not observed at 48 and 72 hrs under the same experimental conditions (Figures 4.16 b and c).

Secondly, the cytoprotective effect of the plant extract on lymphocyte cells exposed to mycotoxins OTA and FB_1 was determined. The MTT assay is normally expected to show a decrease in metabolic activity via decreasing cell mitochondrial activity when cells were exposed to the tested mycotoxins. The results also showed some inconsistent changes in % cell viability unlike the observed progressive cell viability decrease with increasing exposure time to the toxins as previously observed by Mwanza *et al*. (2009). In addition, it was not possible to correlate the cytotoxic response of the lymphocytes to the concentration of mycotoxins as earlier reported by Maenetje *et al*. (2008). Simply, this was because of the extra variable of plant extract added in this study.

Furthermore, the elevated % cell viability seemingly observed with the combined effect of both the extract and the mycotoxins was rather puzzling, since it was obvious that either the extract or the mycotoxins alone demonstrated some cytotoxic responses on the lymphocytes. These experiments were repeated several occasions and each outcome demonstrated no correlation or unexplainable inconsistencies, possibly additive effects of the two variables on the lymphocytes could explain the high % cell viabilities observed in the study (Figures 4.16 b and c). Yet, the additive effect did not account for the observed low % cell viability seen in isolated cases.

Thirdly, observed cell viability upon combining both plant extracts and mycotoxin was expected to vary over time of exposure and as found, there was no consistent pattern to demonstrate plant extracts inhibited the effects of mycotoxins on cell viability. A logical explanation may be that the synergistic or additive effects that have been reported to occur as a result of mycotoxin exposure to cells (Mwanza *et al*., 2009;

Mwanza and Dutton, 2014) produced false positives. This is such that one could seemingly assume that the plant extracts in study provided some protective effects against the tested mycotoxins.

It was also noted that FB_1 when compared to OTA, had a lower cytotoxic activity presenting a higher % cell viability (Figure 4.16) similar to the observation made by Mwanza *et al*. (2009). These findings agree with those from other studies that reported on cell stimulatory effects instead of decreased cell viability after exposure to mycotoxins (Keck and Bodine, 2006). Thus, it becomes even more difficult to determine whether % cell viability seen was attributed to herbal protection or the mycotoxin stimulatory effects. However, it becomes a different scenario *in vivo* where metabolic transformation may either enable the parent compounds to be more toxic or less (Wan *et al*., 2013).

CHAPTER SIX

CONCLUSION AND RECOMMENDATION

6.1 Conclusion

The antifungal activity displayed by *Piptadeniastrium africanum* against *A. flavus*, *A. parasiticus* and *A. niger* is relatively impressive but were limited as the crude extracts also contained other non-biologically active plant components. It is thus necessary to isolate and characterize those active compounds responsible for the conferred antifungal activity observed in this study. In addition, further studies on whether this plant could also inhibit mycotoxin production would be valuable. As demonstrated by Kouadio *et al*. (2013), *Lycopersicone sculentum* leave extract with antifungal activity also inhibited mycotoxin production. This could shed more lights on the potential antimycotoxin application of principally *P. africanum* in preventing fungal crop infestations and possibly, mycotoxin biosynthesis in feeds.

The 2D GCxGC-TOF/MS data generated herein showed the presence of several compounds similar to those found in the hint library which can be linked to specific classes of phytochemicals exhibiting desirable medicinal properties (antifungal, antioxidant, anti-inflammatory, anticarcinogenic, etc.). There were a few compounds with properties that suggest caution in the use of these extracts has to be noted. For example, the phenyl epoxide 2, 2'-bioxirane occurred at a high content of 5.34 and 0.69% in *L. leonurus* and *P. africanum* extracts, respectively, described as a chemical intermediate in the preparation of erythriol and pharmaceuticals (Clayton and Clayton, 1994) is anticipated to be carcinogenic (NCI, 2015). Yet, this plant has been acclaimed in traditional medicine, therefore, more research has to be conducted on the phytochemical compositions of medicinal plants to justify their use and avoid concomitant exposure to some toxic components present in these extracts. However, a concise further screening and fractionation may reveal those pure compounds that could be used in the development of the proposed feed additives.

The EPR spectroscopy studies on the antioxidant activities of the 3 plant extracts *M. longifolia*, *L. leonurus*, and *P. africanum* revealed significant antioxidant activity, which was not drastically altered by exposure to 2 hrs UV irradiation. The *P. africanum* extract had the most superior antioxidant activity even when compared to the Bulgarian leave extracts of *Haberlea rhodopensis* (Grigorov *et al*., 2014). Despite

this, contradictory results were expressed by the *ex vivo* data which revealed that *P. africanum* also possessed pro-oxidant properties as seen by the high levels of ascorbly (•Asc) and reactive oxygen species (ROS) radicals in the liver and brain homogenates of mice treated with this extract. The concentration dependent antioxidant activity observed in the 3 plant extracts studied *in vitro* herein substantially requires some *in vivo* studies essentially because, these extracts are intended for inclusion in animal feeds which is a real situation in the field. Solving the problem with an excessive supply of antioxidants may results in a refractory response (Spasojević *et al*., 2011), which would be undesirable.

Cellular response to oxidative stress is dependent upon the enzymatic and non-enzymatic antioxidant defences in the cell, the type of oxidant, nature of oxidative stress induced as well as dose and exposure time (Kalaiselvi *et al*., 2013). Thus, the cytotoxicity studies were not sufficient in giving conclusive results on the effect of the tested *M. longifolia*, *L. leonurus* and *P. africanum* extracts on cells exposed to OTA and FB_1. However, OTA was more toxic to exposed cells than when FB_1 was tested in which the plant extracts could not improve or prevent the cytotoxic effects expressed by OTA. The question whether the antioxidant function or the cytotoxic property could be used as a criterion on the adequacy of these plant extracts as feed additives or not, remains unanswered.

6.2 Recommendation

The potential in formulating and applying herbal plant components as feed additives or as biotechnological products require the following considerations. First of all, experiments that mimic gastric and intestinal environments, consideration of whether the proposed additive can be measured on the basis of a positive change in performance or reduction of the secondary effects due to mycotoxin exposure. Secondly, *in vivo* experiments should be designed that accommodate testing the proposed additives on target organ protection (TOP) and animal performance (body weight gain, feed consumption capabilities and feed efficiency). These in turn are influenced by the presence of enzymes, beneficial microbiota and other immunological responses that may increase the occurrence of false positives. Thus, it is important to confirm this via performing both *in vivo* and *in vitro* experiments, so

that the proposed herbal product can be potentially considered to have real protection (Douglas, 2013).

Again, the efficiency of herbal additives depends on their volatility, heat stability and pH sensitivity, which influence their retention and activity in stored and processed foods (Atroshi *et al*., 2002). These are some of the important factors that should be considered in drawing a conclusive analysis of the overall potency of the proposed herbal additives, though these are beyond the scope of this study.

Sustainable production of the proposed feed additives would also require alternative biotechnological production of specific plant secondary metabolites or active components. This aspect cannot be over emphasized, since it was obvious with the low extraction yields of crude extracts, which become even lower after fractionation to obtain a pure target compound. In addition, there is a need to comply with attempts made by the Directive of Natural Resources of South Africa to protect and conserve the plant species used for extraction. Further studies may focus on plant biotechnology tissue culture measures or cultivation of the plant species under artificial conditions, to source the desired secondary metabolites. If the cultivation route is followed, it may influence the concentration of these antioxidant compounds which is dependent on the type of soil. Therefore, a study of plant extracts from different sources would aid in determining which sources to use for the best yield. Ideally, chemical enhancement or chemical synthesis may be the appropriate approach.

An assessment that would focus on administering the developed product may involve pilot studies on for example, tolerable intake levels of the developed product as well as the highest level of nutrient intake likely to pose no health risk. A recommended dietary allowance intake level that is sufficient to meet the nutrient requirements (US Institute of Medicine Food and Nutrition Board, 1998) for chicks/pigs would be required. The estimated average intake levels to meet the requirements of each age group of animals and an adequate intake level based on experimentally observed approximations or estimates of nutrient intake (Institute of Medicine (US) Food and Nutrition Board, 2001) would also be necessary.

In a case where the proposed feed additive does not classically meet the dietary antioxidant criteria but acts by aiding or influencing biochemical processes that

reduce oxidative stress linked to OTA and FB_1, traditional development of drugs from natural sources could follow. These could include structure-activity relationships (SAR), synthesis of analogues, mechanism of action studies, and the design and synthesis of drug structures (Chibale, 2005). Also, *in vitro* studies on the active components, i.e., bioavalabity and solubility; efficacy over a period of time; antagonistic, synergistic, additive effects; and possible, molecular modifications for optimum activity are necessary. In addition, models similar to those designed by Devreese *et al*. (2013) that evaluate the efficacy and drug interactions of the proposed additives with the target organs, can be developed and applied to address some of these concerns.

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APPENDICES

APPENDIX I: Phytochemical analysis

1.1 Extraction efficacy

Table A1.1: Extraction efficacy of *M. longifolia*, *L. leonurus* and *P. africanum*

Plant sample	Solvent[a]	Yield (g)	% Yield
M. longifolia	Hex	0.23	1.15
	DCM	0.46	2.3
	EtAc	0.48	2.4
	MeOH	1.6	8
L. leonurus	Hex	0.22	1.1
	DCM	0.41	2.05
	EtAc	0.67	3.35
	MeOH	1.45	7.26
P. africanum	Hex	0.06	0.3
	DCM	0.14	0.72
	EtAc	0.15	0.78
	MeOH	1.32	6.6

[a]Hex: hexane; DCM: dichloromethane; EtAc: etylacetate; & MeOH: methanol

The R_f values of each plant extract was determined using the equation described in Chapter 3 Section 3.3.3.1. Table A1.2 below displays the results.

Table A1.2: R_f values for *M. longifolia*, *L. leonurus*, and *P. africanum*

M. longifolia Solvents	R_f values								
	0.18	0.22	0.32	0.36	0.43	0.46	0.52	0.87	0.97
Hex	-	-	+	+	+	+	+	+	+
DCM	+	+	+	+	+	+	+	+	+
EtAc	+	+	+	+	+	+	+	+	+
MeOH	-	-	+	+	+	+	+	+	+

L. leonurus

Solvent	0.17	0.18	0.24	0.26	0.3	0.36	0.38	0.41	0.5	0.6	0.72	0.9	0.94	0.98
Hex	+	+	+	+	+	+	-	+	+	-	-	+	-	+
DCM	+	+	+	+	+	+	-	+	+	+	-	+	+	+
EtAc	+	+	+	+	+	+	-	+	+	+	+	+	+	+
MeOH	-	-	-	-	-	+	+	+	+	-	-	+	-	+

P. africanum Solvents	0.04	0.06	0.08	0.19	0.32	0.35	0.36	0.38	0.45	0.49
Hex	-	+	-	-	-	+	-	-	-	-
DCM	-	+	+	+	+	+	+	+	-	-
EtAc	-	+	+	+	+	+	+	+	-	+
MeOH	+	+	-	-	-	-	+	-	-	-

Key: presence (+) or absence (-) of a band/spot

1.2 2D GCxGC-TOF/MS

The total ion chromatogram of each extract is a representation of the peaks/spots separated by 2D GCxGC-TOF/MS. The assignment of each peak to the specific compound it represents requires expertise and skill thus the different peaks were not assigned.

Figure A1.1: Total ion chromatogram (TIC) of *M. longifolia* methanol extract

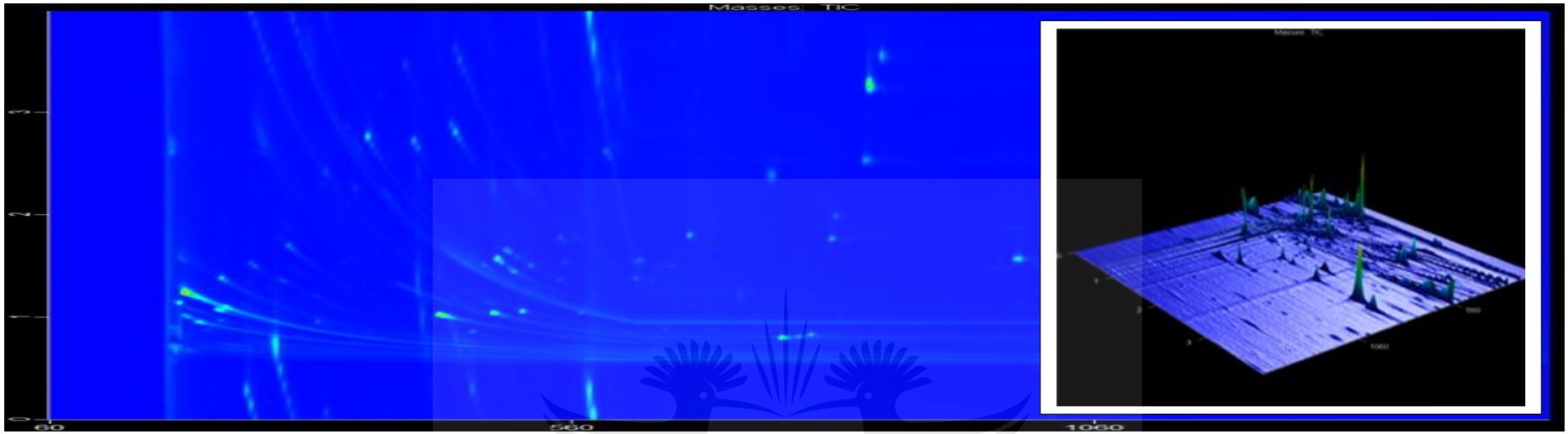

Figure A1.2: Total ion chromatogram (TIC) of *L. leonurus* methanol extract

Figure A1.3: Total ion chromatogram (TIC) of *P. africanum* methanol extract

Table A1.3: Some of the properties of the 6 compounds identified from ***M. longifolia*** methanol extract

Fig no.	Name of Compounds	Nature	Properties	Reference
1	á-Pinene	Terpene	Antioxidant, antimicrobial	Silvério *et al*., 2013
2	I-Menthone	Monoterpene	Flavor and derivative for menthol	Kamatou *et al*., 2013
3	Apocynin	Monoterpene	Anti-inflammatory, Antioxidant	van den Worm *et al*., 2001
4	Naphthalene,1,2,3,5,6,8a hexahydro-4,7-dimethyl-1(1-methylethyl)-, (1S-cis)-	Sesquiterpene	Antifungal	Wang *et al*., 2011
5	Camphene	Terpenoid	Antioxidant and cytoprotective activity	Tiwari and Kakkar, 2009
6	à-Terpineol	Monoterpene alcohol	Anticancer	Hassan *et al*., 2010

Table A1.4: Some of the properties of the 6 selected compounds in ***L. leonurus***

Fig no.	Name of Compounds	Nature	Properties	Reference
1	1-Methyl-pyrrolidine-2-carboxylic acid	Carboxylic acid	Potential of interfering with aflatoxin synthesis at transcription level	Murugan *et al.*, 2013
2	5-Hydroxypipecolic acid	Imino acid	For synthesis of anticancer drug XV710	Vranova *et al*., 2013
3	Cinnamaldehyde, (E)-	Monoterpene	Antioxidant, anti-inflammatory, anticancer	Rao and Gan, 2014
4	2,4-Dihydroxy-2,5-dimethyl-3(2H)-furan-3-one	Furanone	Antioxidant	Schwab, 2013
5	2-Caren-10-al	Monoterpene	Antioxidant, antimicrobial	Hajlaoui *et al*., 2010
6	2H-Pyran-2,6(3H)-dione	Monoterpene	Anticancer	Kranjc and Kocevar, 2013

Table A1.5: Some of the properties of the compounds in ***P. africanum*** methanol extract

Fig no.	Compound in *P. africanum*	Nature	Properties	References
1	Resorcinol	Phenol	antioxidant food additive	EFSA, 2010b
2	N,N-Dimethylglycine	Amino acid glycine	Anti-stress nutrient, anti-inflammatory, antibacterial and nutritional supplement	Natural Medicines Comprehensive Database, 2015
3	Indole	Aromatic	Antimicrobial, main component in anticancer, antidepressant drugs	Biswal *et al*., 2012
4	Phenol,2,6-dimethoxy-4 (2-propenyl)-	Phenylpropanoid	Flavourant	Burdock, 2001
5	Vanillin	Methoxyphenol	Anti-inflammatory activity	Murakami *et al.*, 2007
6	Pantolactone	Lactones	Feed additive	Hilterhaus and Liese, 2007

Table A1.6: Some of the properties of the 12 compounds that were present in more than one plant extract

Fig no.	Plant Present	Name of Compound	Nature	Properties	Reference
1	a, b, c	Phenol, 2,6-dimethoxy	Alkylated phenol	*Neofusicoccum ribis*, *Diplodia seriata* mycelium growth inhibition – antifungal activity	Phenol, 2,6-dimethoxy (n.d)
2	a, b	Phytol	Diterpene	anti-cancer, anti-diabetic, anti-inflammatory, anti-oxidant activity and antimicrobial activity	Pejin *et al*., 2014
3	a, b, c	n-Hexadecanoic acid	Palmitic acid	Antioxidant, flavor	Prabu *et al*., 2013
4	a, b, c	2-Methoxy-4-vinylphenol	phenol	Flavoring agent	Anonymous (n.d)
5	b, c	2,2'-Bioxirane	Phenyl epoxide	Chemical intermediate and in the preparation of erythritol and pharmaceuticals	Clayton and Clayton, 1994
6	a, b, c	4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-	Flavonoid	Antioxidant, anti-inflammatory	Yu *et al*., 2013
7	a, b	2-Propanone, 1-hydroxy-	Aliphatic ketol	synthesis of imidazoles acting as potent and orally active antihypertensive agents	Anonymous (n.d)
8	a, b	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	Terpene alcohol	Antimicrobial and anti-inflammatory	Rajeswari *et al*., 2012
9	a,c	Furfural	Cyclic aldehyde	Fungicide, Biocide, Nematicide	Anonymous (n.d)
10	a,b	Pulegone	Monoterpene	Flavoring agent	
11	a, b, c	1,2-Cyclopentanedione	Monoterpene	good binding affinity for aldose reductase the rate limiting enzyme responsible for triggering the pathogenesis of diabetic cataract	Krishnamoorthy *et al*., 2013
12	a, b	á-D-Glucopyranose, 1,6-anhydro-	Sugar moiety	for the preparation of rifamycin S, indanomycin, thromboxane B2, (+)-biotin, tetrodotoxin, quinone, macrolide antibiotics and modified sugars.	Anonymous (n.d)

Key: a: *M.longifolia*; b: *L.leonurus*; c: *P.africanum*

APPENDIX II: Biological analysis
2.1 Agar diffusion assay

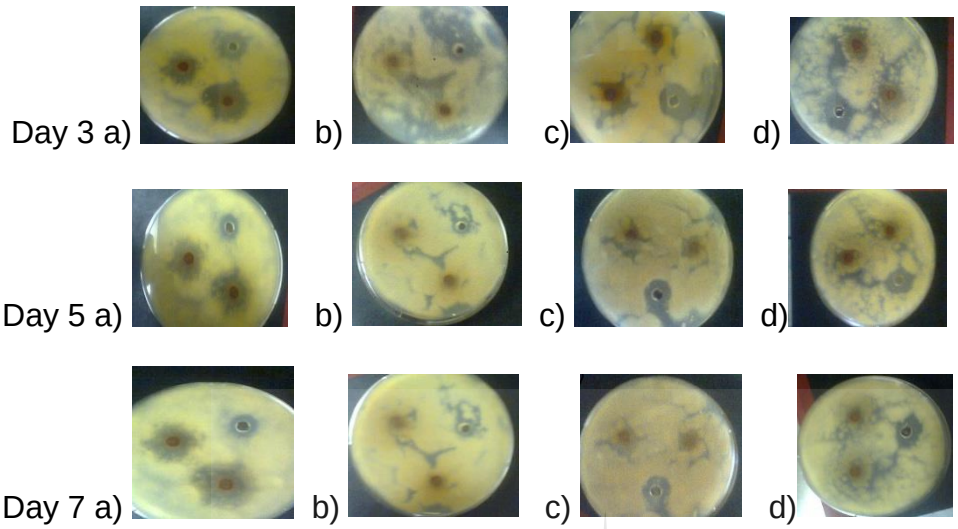

Figure A2.1: Plates showing antifungal activity (growth inhibitory zone) of *P. africunum* extract against a) *A. niger* b) *A. fumigatus* (no zones of clearance except on the AmB), c) *A. flavus* and d) *A. parasiticus* on Day 3, 5, and 7

APPENDIX III: Antioxidant activity EPR studies

Table A3.1: The% DPPH scavenging activity of *M. longifolia* from 0.1% stock solution

M. longifolia (µl)	% DPPH scavenging activity
10	17
20	27
30	40

Table A3.2: The **%** DPPH scavenging activity of 1 mg/ml *L. leonurus* methanol extract from 0.1% stock solution

L. leonurus (µl)	% DPPH scavenging activity
10	27
20	46
30	69
50	86
100	99

Table A3.3: The % DPPH scavenging activity of 1 mg/ml *P. africanum* methanol extract from 0.1 and 0.0125% stock

Sample *P. africanum* (µl)	%DPPH scavenging activity
0.1% stock solution	
10	99.35
0.0125% stock solution	
10	45.06
20	61.99
30	78.71
50	98.17
100	99.56

Table A3.4: The antioxidant activities of *P. africanum* measured after UV irradiation and exposure to high ultrasonic waves

Sample (µl)	% DPPH scavenging activity
After 2 hrs UV-irradiation stock 0.1%	
10	98.33
20	99.99
After 2 hrs UV-irradiation 0.0125%	
10	54.53
20	62.29
30	77.72
50	86.89
100	98.76

Table A3.5: The % DPPH scavenging activity of 0.0125% concentration of *P. africanum* before and after UV irradiation at 10, 30 min and 24 hrs after incubation in the dark

Sample	% DPPH scavenging activity after 10 (min)	30 (min)
Before irradiation	67.97	37.14
After 2 hrs UV irradiation	84.34	96.66

Sample	% DPPH scavanging activity 10 µl	50 µl
After 2 hrs UV irradiation	8.51	50.46
After 2 hrs UV irradiation, and at 24 hrs after irradiation	43.51	53.96

Table A3.6: Levels of •Asc radicals in the liver and brain homogenates after exposure to *P. africanum* methanol extract

•Asc radicals (DI/N)			
Liver		Brain	
Control	Sample	Control	Sample
0.1695	0.5751	0.352	0.9228
0.1716	0.564	0.3342	0.8443
0.1928	0.6489	0.3416	1.072
0.1983	0.6686	0.3223	1.003
0.18305 ± 0.01	0.61415 ± 0.05	0.3375 ± 0.01	0.9605 ± 0.09

Values in the last row are means (n=4) ± standard deviation (SD).
Means are not significantly different (p≥0.05)

Table A3.7: Levels of ROS/PBN radicals in liver and brain homogenates after exposure to *P.africanum* methanol extract

ROS/PBN. radicals (DI/N)			
Liver		Brain	
Control	Sample	Control	Sample
0.6275	2.098	0.348	2.115
0.792	2.014	0.4164	1.537
0.7451	1.713	0.246	1.453
0.5153	1.003	0.2566	1.825
0.6699 ± 0.12^{a}	1.707 ± 0.50^{b}	0.3167 ± 0.08^{a}	1.7325 ± 0.30^{b}

The values in the last row are means (n=4) ± standard deviation, at 0.05 level
the means are significantly different

APPENDIX IV: Cytotoxicity studies

Table A4.1: Percentage viability of human lymphocytes exposed to concentrations of *M. longifolia* (M), *L. leonurus* (L), and *P. africanum* (P) extracts and mycotoxins (OTA and FB_1) after a) 24 hrs b) 48 hrs and c) 72 hrs intervals

24 hrs																							
					20 µg/ml						40 µg/ml						80 µg/ml					
					OTA			FB1			OTA			FB1			OTA			FB1		
		P	M	L	P	M	L	P	M	L	P	M	L	P	M	L	P	M	L	P	M	L
PC	100±0.00^{a}																					
STD 1	86±0.02^{a}																					
STD 2	74±0.19^{c}																					
2 mg/ml		50±0.01^{a}	55±0.02^{a}	98±0.01^{a}	63±0.00^{a}	53±0.00^{a}	105±0.02^{a}	54±0.06^{b}	60±0.03^{a}	253±0.17^{c}	121±0.01^{a}	53±0.08^{b}	257±0.04^{a}	196±0.20^{c}	90±0.20	90±0.20^{c}	79±0.02^{a}	42±0.00^{a}	252±0.02^{a}	79±0.11^{b}	116±0.28^{d}	66±0.09^{b}
1mg/ml		42±0.01^{a}	37±0.01^{a}	75±0.02^{a}	36±0.00^{a}	58±0.03^{a}	105±0.04^{a}	234±0.15^{b}	164±0.09^{b}	264±0.60^{g}	42±0.03^{a}	42±0.03^{a}	276±0.08^{b}	118±0.01^{a}	228±0.40	228±0.40	97±0.07^{b}	36±0.00^{a}	226±0.02^{a}	84±0.00^{a}	192±0.16^{c}	116±0.04^{a}
0.1mg/ml		42±0.01^{a}	47±0.02^{a}	87±0.09^{b}	236±0.02^{a}	26±0.00^{a}	257±0.04^{a}	206±0.43^{e}	142±0.36^{e}	116±0.41^{c}	53±0.00^{a}	247±0.04^{a}	223±0.02^{a}	116±0.17^{c}	260±0.20	260±0.20	51±0.01^{a}	31±0.00^{a}	210±0.14^{b}	215±0.01^{a}	124±0.37^{e}	134±0.14^{b}

48 hrs																							
					20 µg/ml						40 µg/ml						80 µg/ml					
					OTA			FB1			OTA			FB1			OTA			FB1		
		P	M	L	P	M	L	P	M	L	P	M	L	P	M	L	P	M	L	P	M	L
PC	100±0.00^{a}																					
STD 1	68±0.01^{a}																					
STD 2	75±0.16^{c}																					
2 mg/ml		73±0.00^{a}	98±0.03^{a}	108±0.00^{a}	270±0.03^{a}	290±0.01^{a}	100±0.01^{a}	112±0.17^{c}	69±0.00^{a}	171±0.01^{a}	200±0.18^{c}	250±0.04^{a}	270±0.09^{b}	65±0.03^{a}	154±0.11^{b}	37±0.01^{a}	265±0.50^{f}	270±0.03^{a}	240±0.03^{a}	87±0.01^{a}	112±0.04^{a}	57±0.54^{f}
1mg/ml		57±0.54^{f}	96±0.04^{a}	82±0.01^{a}	155±0.20^{c}	260±0.04^{a}	126±0.03^{a}	37±0.01^{a}	109±0.01^{a}	96±0.03^{a}	210±0.38^{f}	235±0.03^{a}	260±0.04^{a}	72±0.04^{a}	56±0.41^{f}	59±0.11^{b}	270±0.09^{b}	260±0.16^{c}	150±0.02^{a}	64±0.76^{j}	109±0.11^{b}	35±0.16^{c}
0.1mg/ml		51±0.01^{a}	95±0.01^{a}	135±0.17^{c}	220±0.60^{g}	245±0.60^{g}	290±0.28^{d}	52±0.1^{b}	94±0.01^{a}	47±0.01^{a}	205±0.01^{a}	270±0.03^{a}	220±0.12^{b}	52±0.04^{a}	135±0.40^{f}	69±0.54^{f}	285±0.30^{d}	90±0.09^{b}	205±0.01^{a}	91±0.41^{f}	21±0.54^{f}	44±0.01^{a}

72 hrs																							
					20 µg/ml						40 µg/ml						80 µg/ml					
					OTA			FB1			OTA			FB1			OTA			FB1		
		P	M	L	P	M	L	P	M	L	P	M	L	P	M	L	P	M	L	P	M	L
PC	100 ±0.00^{a}																					
STD 1	72±0.17^{c}																					
STD 2	64±0.34^{d}																					
2 mg/ml		115±0.44^{f}	90±0.02^{a}	82±0.00^{a}	61±0.00^{a}	51±0.00^{a}	46±0.00^{a}	120±0.12^{b}	175±0.00^{a}	165±0.18^{c}	107±0.02^{a}	49±0.02^{a}	51±0.01^{a}	119±0.55^{f}	120±0.48^{f}	115±0.15^{b}	59±0.01^{a}	111±0.02^{a}	106±0.32^{d}	105±0.32^{d}	160±0.11^{b}	165±0.04^{a}
1mg/ml		95±0.04^{a}	105±0.01^{a}	81±0.03^{a}	56±0.14^{b}	54±0.00^{a}	51±0.02^{a}	150±0.03^{a}	190±0.35^{d}	170±0.40^{e}	66±0.01^{a}	46±0.02^{a}	43±0.02^{a}	135±0.08^{b}	145±0.10^{b}	113±0.30^{d}	51±0.02^{a}	128±0.01^{a}	95±0.34^{d}	101±0.12^{b}	138±0.37^{e}	135±0.00^{a}
0.1mg/ml		105±0.00^{a}	85±0.00^{a}	85±0.04^{a}	130±0.05^{a}	43±0.01^{a}	46±0.03^{a}	190±0.07^{b}	140±0.34^{d}	190±0.14^{b}	38±0.01^{a}	146±0.02^{a}	92±0.34^{d}	135±0.03^{a}	180±0.29^{d}	105±0.28^{d}	49±0.01^{a}	102±0.01^{a}	46±0.01^{a}	110±0.15^{b}	145±0.02^{a}	110±0.01^{a}

Means + standard deviation (SD) within the same column with different superscripted letters significantly different ($p<0.05$)