MYCOTOXIGENIC FUNGI AND MULTIMYCOTOXIN CONTAMINATION IN MEDICINAL PLANTS SOLD IN THE FREE STATE PROVINCE, SOUTH AFRICA.

by

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DECLARATION OF INDEPENDENT WORK

I, JULIUS NDORO, identity number _____ and student number _____ do hereby declare that this research project submitted to the Central University of Technology, Free State for the Degree of MASTER OF HEALTH SCIENCES IN ENVIRONMENTAL HEALTH, is my own independent work and complies with the Code of Academic Integrity, as well as other relevant policies, procedures, rules and regulations of the Central University of Technology, Free State; and has not been submitted before to any institution by myself of any other person in fulfilment (or partial fulfilment) of the requirements for the attainment of any qualification.

SIGNATURE OF STUDENT

05 June 2023
DATE

DEDICATION

I dedicate this thesis to my parents, Mr and Mrs Ndoro. I am grateful for the love, encouragement, support in countless ways and prayers that kept me going on this journey.

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SUMMARY

MYCOTOXIGENIC FUNGI AND MULTIMYCOTOXIN CONTAMINATION IN MEDICINAL PLANTS SOLD IN THE FREE STATE PROVINCE SOUTH AFRICA

South Africa has a long history and extensive use of medicinal plants in traditional medicine, but relatively few studies have been conducted on their contamination with moulds and mycotoxins. The purpose of the present study was to characterise medicinal plant trade and determine the extent of fungal and mycotoxin contamination among frequently sold medicinal plants in the Free State Province not yet reported before. A market survey was conducted using semi-structured interviews across 29 locations with 48 respondents. Fungi species were isolated as single colonies morphologically and identified using molecular techniques. Mycotoxin analysis of samples was done using Ultra High-Pressure Liquid Chromatography-Tandem Mass Spectrometry (UPLC-ESI-MS/MS) for the simultaneous detection of AFB_1, DON, FB_1, FB_2, FB_3, NIV, OTA, and ZEN.

The trade in medicinal plants was mainly dominated by street vendors and *muthi* shops made up mainly of Sotho, Zulu, Ndebele ethnicities. Traders were mostly men who worked full time with no other income sources and had been in business for 0.5 years up to 26 years. The mean monthly income for men was ZAR 1 825, and for women was ZAR 760. A total of 165 ethno-species belonging to 48 families were the most frequently sold medicinal plants. Salient families for the medicinal plant trade in the province were *Asteraceae,* with 13 species, followed by *Liliaceae (5)* and *Fabaceae (3).* The dominant species were *Hypoxis latifolia, Dicoma anomala, Helichrysum odoratissimum, Elephantorrhiza elephantina, Tulbaghia alliacea,* and *Pentanisia prunelloides*.

Thirty-four medicinal plant samples were purchased from street vendors and *muthi* shops in the Free State Province. Approximately 26% of the samples had no fungi contamination, whilst 74% were only contaminated by one or more fungal species. The fungal load in positive samples purchased from street vendors ranged from 1 ×

10^6 to 8.4 × 10^7 with a mean of 2.55 × 10^7 CFU/g. Whereas for *muthi* shops, the study reported a mean fungal load of 2.33 ×10^7, ranging from 1 × 10^6 to 8.4 × 10^7 CFU/g. For all positive samples, the fungal load was above 1 × 10^6 CFU/g (above the WHO limit).

A total of 54 fungal isolates were recovered, comprising of 17 species. Species belonging to *Penicillium, Aspergillus, Fusarium,* and *Purpureocillium* were the most dominant in the medicinal plant samples. About 59% of the fungal isolates screened for their ability to produce mycotoxins were toxigenic, whilst 41% did not produce any detectable quantities of mycotoxins under investigation. All fungal isolates did not produce, nivalenol, ochratoxin A and zearalenone in culture.

Ten out of 34 samples tested positive for mycotoxins as follows; AFB_1(10%); OTA (10%), FB_1 (30%), FB_2 (50%), FB_3 (20%) and ZEN (30%). Mean concentration levels ranged from AFB_1 (15 µg/kg), OTA (4 µg/kg) FB_1 (7-12 µg/kg), FB_2 (1-18 µg/kg), and FB_3 (1-15 µg/kg), ZEN (7-183 µg/kg). Multimycotoxin contamination was observed in 30% of the positive samples with fumonisin derivatives. The concentration of AFB_1 reported in this study is above the South African permissible limit for AFB_1 (5 µg/kg). Fumonisin concentration did not exceed the South African limit set for raw maize grain (4000 µg/kg of FB_1 and FB_2). ZEN and OTA are not regulated in South Africa but reported concentrations were below EU Commission-set limits.

The study findings indicate the prevalence of mycotoxin contamination in frequently traded medicinal plants, mycotoxins pose a significant health risk to consumers due to their additive or synergistic health effects. Routine monitoring of multiple mycotoxin contaminations, human exposure assessments using biomarker analysis, and establishing regulations and standards.is essential. Therefore, strategies to mitigate mycotoxin contamination should be enacted at major markets, traditional healers, local suppliers, *muthi* shops and street vendors.

TABLE OF CONTENTS

LIST OF APPENDICES

a) University of Free State Health Sciences Research and Ethics Committee approval.
b) University of Free Sate Environment and Biosafety Ethics Committee approval.
c) Informed consent form.
d) Questionnaire: Interview guide.
e) Supplementary data: list of medicinal plants sold in the Free State province.
f) Study photographs.
g) Manuscript published in *Toxins*. ISSN 2072-6651.

LIST OF FIGURES

LIST OF TABLES

LIST OF PHOTOGRAPHS

ABBREVIATIONS

AFB_1: Aflatoxin B_1

aw: Water activity

BLAST: Basic Alignment Search Tool

DB: Dry Basis Moisture

DNA: Deoxyribonucleic acid

DON: Deoxynivalenol

EC: European Union Commission

ESI: Electron Spray Ionisation

FAO: Food Agricultural Organisation

FB_1: Fumonisin B_1

FB_2: Fumonisin B_2

FB_3: Fumonisin B_3

FS: Free State

GC: Gas Chromatography

GC/MS: Gas Chromatography-Mass Spectrometry

gDNA : Genomic DNA

GMP-Good Manufacturing Practices

HPLC : High-Pressure Liquid Chromatography

i.d: Internal diameter

IARC: International Agency for Research on Cancer

KZN: KwaZulu Natal

LCMS : Liquid Chromatography-Mass Spectrometry

LCMS/MS: Liquid Chromatography-Tandem Mass Spectrometry

MS: *Muthi* Shop

MW: Molecular weight

NIV: Nivalenol

OTA: Ochratoxin A

PCR: Polymerase Chain Reaction

SV: Street Vendor

TPC: Total polyphenol content

TFC: Total flavonoid content

USD: United States Dollars

v/v: Volume /Volume

WHO: World Health Organisation

ZAR: South African Rand

ZEN: Zearalenone

µl: Microlitre

µM: Micromolar

DEFINITION OF TERMS

Medicinal Plant: A medicinal plant is defined as "any plant which, in one or more of its organs, contains substances that can be used for therapeutic purposes, or which are precursors for the synthesis of useful drugs" (Sofowora et al., 2013).

***Muthi* shop:** A store which sells processed and unprocessed medicinal plants including herbal mixtures and other traditional medicines from a building or fabricated structure.

Street vendor: Refers to a trader who sells medicinal plants from streets pavements or open space, road not specifically allocated by a local authority**.**

Market: A place where medicinal plants are sold by gatherers, suppliers, wholesalers usually to traders for resale or consumers for direct consumption.

CHAPTER 1
GENERAL INTRODUCTION

1.1 Background

Medicinal plants have been considered an essential source of health and alternative therapeutic resources since ancient times. Many people of different cultures and beliefs depend on indigenous medicinal plants as food and natural drugs to meet their basic health care requirements (Pan et al., 2014). For centuries medicinal plants have been used to treat many diseases and conditions, including cancer, diabetes, tuberculosis, menstrual problems, and stomach illnesses (Mintah et al., 2019). In addition, they have been used as insecticides, pesticides, protective charms, and during divination and/or communicating with ancestors (Petrovoska, 2012; Mintah et al., 2019). Medicinal plants play a crucial role in disease prevention, their promotion and use compliments current prevention strategies under the World Health Organisation (WHO) Primary Health Care Approach (Sofowora et al., 2013).

According to Robinson and Zhang (2011), of the 250 000 higher plant species found on earth, more than 80 000 have at least some medicinal properties. Africa contains between 40 to 45 000 plant species with developmental potential, of which 5 000 species are used for their therapeutic properties. South Africa has around 30 000 flowering plant species, accounting for about 10% of the world's higher plant species (Van Wyk & Prinsloo, 2018). Previous studies have indicated that between 350 and 700 South African plant species are commercially traded due to their medicinal properties (Mander, 1998; Van Wyk & Prinsloo, 2018).

The demand and use of medicinal plants and their preparations have increased over the past few years. This growth is attributed to increased societal acceptability, compatibility, and adaptability with the human body, including perceived fewer side effects (Pan et al., 2014; Miranda & Venacio, 2019). Compared to developed countries, medicinal plant use and trade in Africa is high, around 80%, due to economic, social, and cultural factors (Van Wyk & Prinsloo, 2018).

Fortune Business Insights (2022) estimates the global herbal medicine market to grow from $165.66 billion in 2022 to $347.50 billion by 2029. In South Africa, Mander and Le Brenton (2006) reported an annual consumption of around 700 000 tonnes of plant material worth 150 million US dollars. The global increase in medicinal plant use over the past decades has led to the establishment of urban herbal markets in developing countries dominated by people from rural and urban areas (Van Wyk & Prinsloo, 2018; Williams et al., 2000). Furthermore, the increased consumption of medicinal plants has made their safety and quality a public health concern (Street et al., 2008; Van Wyk & Prinsloo, 2020).

Safety is a critical factor that takes precedence over marketing, trade, and usage of herbal medicines (Calixto, 2000; Miranda & Venacio, 2019). In particular, the quality of herbal products depends on the safety and efficacy of the herbal content concerning the inherent chemical components. Moreover, medicinal plants may be contaminated with fungi and mycotoxins during production, harvesting, and post-harvesting, losing their physical characteristics and efficacy (Ashiq et al., 2014). The fungal genus of *Aspergillus, Fusarium, Penicillium, Alternaria,* and *Claviceps* has been reported as the main fungal species causing regular and problematic contamination of herbal medicines (Kneifel et al., 2002; Zain, 2011; Ashiq et al., 2014; Altyn & Twaruzek, 2020). Fungal contamination is more prevalent in tropical and subtropical climates, as high temperatures and moisture content lead to fungal growth and toxin production (Kneifel et al., 2002; Cotty & Ramon, 2007; Milani, 2013).

Fungal species have continuously raised global food safety concerns due to their abundance in the environment, potential to colonise food items and cause either physical damage or produce mycotoxins. Mycotoxins are naturally occurring toxic compounds produced by the secondary metabolism of different toxigenic fungal species (Zain, 2011; De Saeger et al., 2016; Greeff-Laubscher et al., 2020;). Worldwide, several studies have reported pre- and post-harvest contamination of medicinal plants with toxigenic fungi and mycotoxins (Aziz et al., 1998, Bugno et al., 2006; Santos et al., 2009; Miranda & Venacio, 2019; Altyn & Twaruzek, 2020).

Mycotoxin exposures have been reported to induce diverse and powerful toxic effects in humans (Fink-Grernmels, 1999; Zain, 2011; Ashiq et al., 2014;). Acute mycotoxicosis cases have been reported, and long-term exposure to low levels of

mycotoxins is a risk factor for diseases such as cancer and stunting in children (Peraica et al., 1999; Shepard, 2008; Wild & Gong, 2010; Lombard, 2014). Mycotoxins present other biological health effects on humans and animals, including but not limited to carcinogenic, teratogenic, oestrogenic, haemorrhagic, immunotoxic, nephrotoxic, and neurotoxic (Peraica et al., 1999; Wild & Gong, 2010; Zain, 2011). Multiple exposures to different mycotoxins can induce various additive, synergistic, or antagonistic toxic effects. However, very little information exists about studies on the combined health risks from multiple mycotoxin exposures (Speijers & Speijers, 2004; De Ruyk et al., 2015; Assunção et al., 2016; Smith et al., 2016; Alassane-Kpembi et al., 2017).

Food and medicine safety and security remain basic human needs in today's rapidly evolving and changing world. The safety of traditional medicines and foods is critical to public health promotion and quality of life. Despite the extent of consumption, demand, and medicinal plant trade and subsequent health risks to the consumers posed by fungi and mycotoxins, there is a dearth of information and studies on fungi and mycotoxin contamination of medicinal plants and their products in South Africa.

1.2 Problem Statement

Access to safe and affordable food and medicines is of paramount importance in maintaining healthy and productive lifestyles. Consumers expect and deserve protection against health risks found in food and medicines (Gardner, 1993; Wang et al., 2020). Foodborne illness is a significant public health problem for developed and developing countries, including economic burdens related to medical costs and lost productivity (Deon et al., 2014; McLinden et al., 2014; Wang et al., 2020). The safety and quality of medicinal plants are essential as they are also susceptible to microbial contamination, just like other food products and agricultural commodities (Zain, 2011; Ekor, 2013, Miranda & Venacio, 2019). Due to fungi's ubiquitous nature, they are the major microbial contaminants of medicinal plants and their products (Kneifel et al., 2002; Pitt & Hocking, 2009). Contamination of medicinal plants by various spoilage and toxigenic fungi may occur pre-and post-harvest (Zain, 2011; Ashiq et al., 2014). Using medicinal plants for generations does not guarantee their safety; therefore,

compliance with safety and quality requirements is vital (Ekor, 2014; Moreira et al., 2014).

Despite the long history and prevalent use of medicinal plants in South African traditional medicine, few studies have been done on their contamination with moulds and mycotoxins compared to the abundant publications on cereals, fruits, and oil seeds (Trucksess & Scott, 2008). The increasing demand for medicinal plants may exacerbate consumer risk of exposure to mycotoxins due to the lack of regular and adequate safety and quality control monitoring (Bugno et al., 2006; Van Wyk et al., 2020). Given the growing market share of medicinal plants and products, there is a need to ensure that they are safe for use to protect consumers from associated health risks (Street et al., 2008; Ekor, 2014; Van Wyk et al., 2020).

The public may continue to use these medicinal plants and their products, unaware of the long-term health effects associated with moulds and mycotoxin exposures (Bugno et al., 2006; Ashiq et al., 2014; Van Wyk et al., 2020). More studies should be conducted to classify the prevalent fungal strains, toxicity, and the level of mycotoxin contamination in commonly used medicinal plants (Zain, 2011; Ashiq et al., 2014). Therefore, the present study characterised medicinal plant trade and determined the extent of fungal and mycotoxin contamination among frequently sold medicinal plants in the Free State province. This information and data are currently unknown in this province.

1.3 Hypothesis

1. Medicinal plants sold in formal and informal markets may be contaminated with toxigenic fungi and by more than one type of mycotoxin.

1.4 Research Aim and Objectives

1.4.1 Aim

The study aimed to characterise medicinal plant trade in the Free State Province, South Africa and assess the occurrence of fungi and mycotoxins in commonly sold medicinal plants.

1.4.2 Objectives

1. To identify the frequently traded medicinal plants in the Free State province markets.
2. To determine the incidence of fungal contamination in market traded medicinal plants.
3. To determine and quantify multiple mycotoxin occurrences among frequently sold medicinal plants.
4. To recommend possible strategies for mycotoxin control in medicinal plants and their products.

1.5 Significance and value of research

The study will contribute to public health safety by providing baseline knowledge that can be used to inform evidence-based strategies to reduce fungal spoilage and subsequent mycotoxin contamination in commonly traded medicinal plants.

1.6 Chapter outline

The study is presented in six sections, as shown in **Figure 1.1. Chapter 1** provides a background on the study showing the increased use of medicinal plants as therapeutic agents and the subsequent growth in commercial trade without routine safety and quality monitoring for consumer protection. **Chapter 2** reviews literature on medicinal plant trade, preservation and storage, fungi spoilage, fungal species and mycotoxins, including identification and analysis methods. Prevention and control methods for fungi and mycotoxin contamination are also discussed. **Chapter 3** focuses on the medicinal plant trade in the Free State province regarding market characteristics, socio-economic factors influencing trade, market dynamics and traded species and samples collected from street vendors and *muthi* shops. Samples purchased were evaluated for fungi and mycotoxin contamination. In **Chapter 4,** purchased samples are prepared for enumeration of fungi contamination using the microdilution method. The isolation and preparation of pure fungal isolates are conducted on Potatoe Dextrose Agar, Water Agar and Malt Extract Agar followed by gDNA extraction and identification using ITS sequencing. Pure isolates are evaluated for their ability to produce mycotoxin on

MEA and analysed using Ultra High-Pressure Liquid Chromatography-Tandem Mass Spectrometry (UPLC-ESI-MS/MS). **Chapter 5** focuses on screening purchased samples for multiple mycotoxin contamination using (UPLC-ESI-MS/MS) for the simultaneous detection of AFB_1, DON, FB_1, FB_2, FB_3, NIV, OTA and ZEN. **Chapter 6** provides the general discussions, conclusions and recommendations for the entire study.

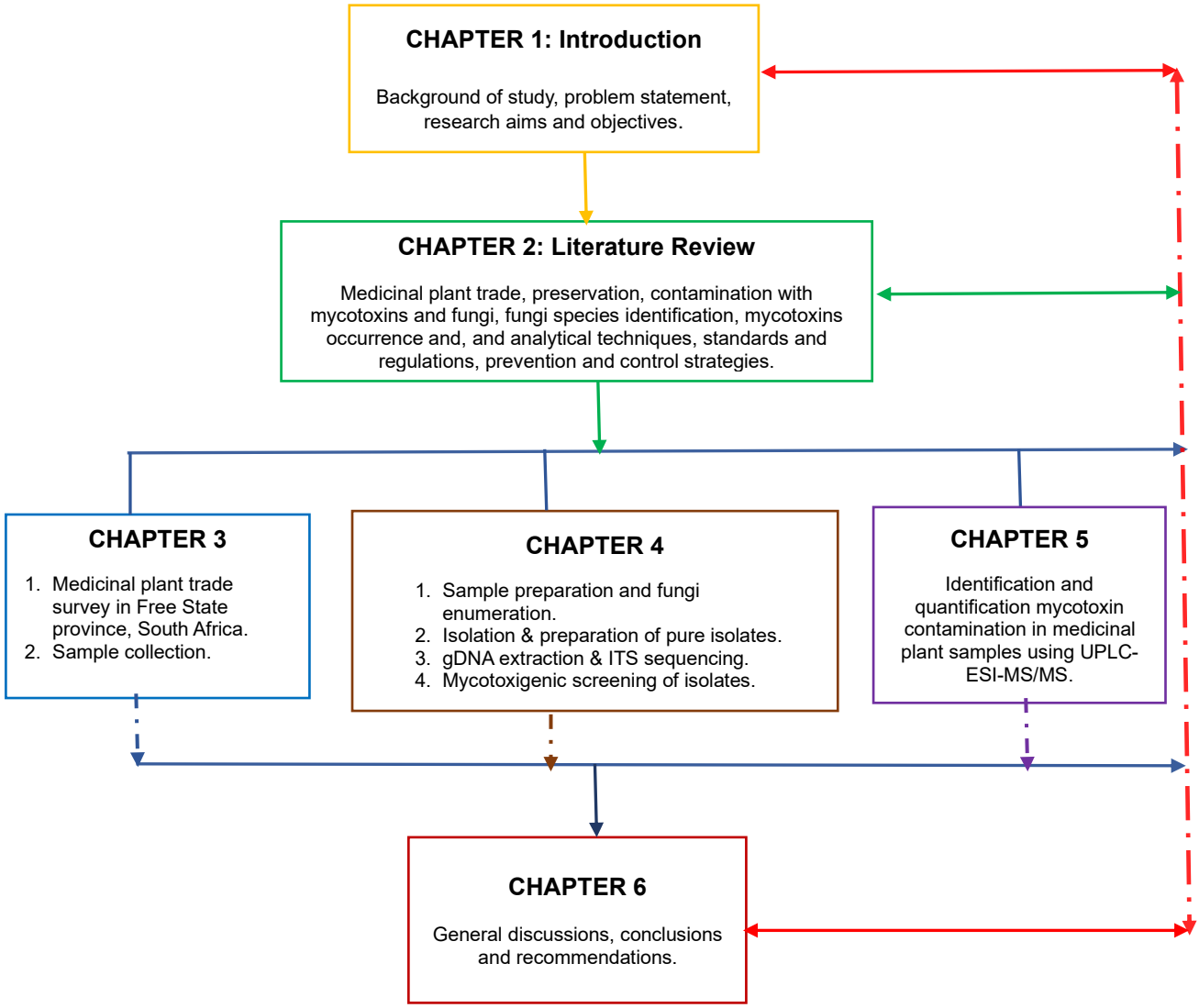

Figure 1.1: Chapter outline demonstrating the relationships between the chapters.

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CHAPTER 2
LITERATURE REVIEW

Abstract

This review summarises the medicinal plant trade in Africa and the factors influencing fungi and mycotoxin contamination. The trade and economic importance of medicinal plant trades has been reviewed, demonstrating their role in primary health care and livelihood. We also provide information on the implications of significant fungi species and mycotoxins affecting medicinal plants. Fungi identification methods and mycotoxin analysis techniques are reviewed, focusing primarily on medicinal plants and their products. The review demonstrates the general studies for mycotoxin contamination in medicinal plants, with only a few studies in South Africa.

Furthermore, the use of genomic sequencing in fungal species identification is becoming widespread. Also, the use of LC/MS-based techniques in fungal identification and quantification is the most preferred analytical technique. Common methods for medicinal plants or herbs preservation and storage have been reviewed, including prevention and control strategies for mould and mycotoxin contamination. Due to the limited studies on mycotoxin contamination in South African medicinal plants, more studies should focus on the frequently traded species.

2.1 Introduction

Medicinal plants have been used for centuries in various cultures as traditional medicines and are sources of modern drugs today (Van Wyk & Prinsloo, 2018). The medicinal plants' therapeutic value is found in chemical substances that create or cause physiological effects on the human body (Mahomoodally, 2013; Lee et al., 2017).

Worldwide, an estimated 60 000 species are used for food, therapeutics, and aromatics (WHO, 2015). Medicinal plants are used extensively in Southern African traditional medicine based on about 3 000 species (Van Wyk & Gericke, 2000).

Southern African medicinal plants used in traditional medicine comprise 215 families, 1 240 genera, and 3 689 taxonomic groups, accounting for about 15% of the regional flora (Arnold et al., 2002). In comparison, Van Wyk (2020) reported that Traditional African Medicine in sub-Saharan Africa consists of 192 out of 254 African families comprising at least 4 576 vascular plant species and 1 518 genera. This indicates that South Africa has the richest medicinally valuable plant population globally. The above studies have shown the importance of medicinal plant species in the health care system and demonstrate the increasing number of medicinal plant species used in traditional medicine.

At least 80% of the world's population is estimated to rely solely or partially on traditional medical remedies for primary health care needs (Ekor, 2014). Whereas 70–95 per cent of the population in Asia, Africa, Latin America, and the Middle East rely on traditional medicine to treat various diseases (Barata et al., 2016). Mander et al. (2007) reported that 72% of black South Africans use traditional medicine services in rural and urban areas contributing to 26.6 million consumers. Dependence on traditional medicine in treating diseases stems from a lack of access to modern healthcare facilities, cultural practices and confidence in traditional approaches. In addition, unnecessary queues in clinics and hospitals and high costs associated with conventional or western medicine (Nxumalo et al., 2011). South Africa is much more developed than other African countries, but the reliance on traditional medicine is still widespread. Furthermore, there is a wide gap between the rich and poor, with the poor making the bulk of the population, hence the reliance on medicinal plants as mentioned before would be typically cheaper and affordable for the wider majority. Therefore, this indicates that medicinal plant use might be significantly higher (probably above 80%) in other less developed countries. Furthermore, the absence of current data on the use of medicinal plants warrants further research to support public health interventions incorporating modern and traditional medicine approaches.

It has been estimated that up to 700 000 tonnes of plant material are consumed annually at a value of approximately 150 million United States Dollars (Wiersum et al., 2006). Mander (1998) reported an earlier annual consumption of approximately 20 000 tonnes of indigenous plant material. An average South African consumer uses traditional medicine at least 4.8 times per year, with a mean mass of 0.157 kg of plant material per treatment (Mander et al., 2007). The role of medicinal plants in primary

healthcare must be acknowledged, considering that the extent of consumption is likely to increase, as evidenced by traditional herbal medicines use during the COVID-19 pandemic (Garcia, 2020; Chali et al., 2021; Lam et al., 2021). Many people are at risk of consuming low-quality medicinal plants and their products; therefore, safety and quality monitoring are of paramount importance in safeguarding public health.

2.2 Methods

An electronic English-restricted search of Google Scholar, Science Direct, and PubMed was undertaken to select literature relevant to medicinal plant trade, fungi, and mycotoxin contamination. The search was limited to published articles from 2015- to December 2020. However, few published articles on mycotoxin contamination in South African medicinal plants were extended to cover the period from 1990 for some review sections. The search began with the terms "South African medicinal plant trade" and "medicinal plant preservation and storage " combined with "safety and quality" and "fungi and mycotoxin contamination". Additional electronic Google Scholar searches using the terms: "fungi species", "fungi identification", "fungi contamination effects", "factors influencing fungi contamination", "mycotoxins", " mycotoxin occurrence in African medicinal plants", "mycotoxins analysis", "impacts of mycotoxins", "prevention and control of mycotoxin contamination", "Microbial limits", "mycotoxins standards and regulations" were undertaken. Books, dissertations, conference papers, technical reports and theses were also consulted on the topics of "medicinal plants trade", "preservation and storage", and "fungi and mycotoxin contamination".

2.3 Trade in medicinal plants and herbal products

Global trade and public demand for medicinal plants have grown in quantity and export value (Vasisht et al., 2006). The global herbal market is estimated at USD 100 billion and is projected to increase annually, significantly contributing to producer countries' economies (Khan & Ahmad, 2019). Delbanco et al. (2017) reported that medicinal plants trade in northern Kenya was based on an annual volume of 55 tonnes per year at a value of USD 25 900. In Gabonese markets, Towns et al. (2014) reported a volume

of 27 tonnes of medicinal plant products worth USD 15 million to be traded annually in main markets from 217 species. A study of Sierra Leon's informal urban markets in three major cities showed that more than 40 plant species were traded with an annual value of USD 64 000, contributed mainly by nine (9) frequently traded species (Jusu & Cuni-Sanchez, 2014). In another study in Tanzania, 61 tonnes of non-powdered medicines valued at USD 344 882 were traded annually in informal herbal medicine markets (Posthouwer, 2015). The trade of herbal medication and the collection of medicinal plants in Africa engenders economic opportunities for resource-poor communities in rural, urban, and marginalised areas (Mander, 1998; Towns et al., 2014; Meke et al., 2017). Medicinal plant trade-economic importance is a primarily understudied area in Africa. However, as shown by the revenue inflows reported by other studies, medicinal plants' trade must be characterised in each country to reflect on the local importance of trade and its current livelihood contribution.

The use and trade of medicinal plants for medicine is no longer restricted to traditional healers but is now part of the South African formal and informal businesses, which has resulted in more people engaging in medicinal plants collection and trade (Dold & Cock, 2002; Van Wyk & Prinsloo, 2018). The medicinal plants' trade is now a source of employment for mainly rural populations, especially women (Katerere et al., 2008; Rasethe et al., 2018). About half a million people in South Africa are directly involved in the medicinal plants’ trade (Afolayan & Adebola, 2004), with at least 133 000 people employed in the trade made up of primarily rural women (Mander et al., 2007). The demand for medicinal plants has been exacerbated by rapid urbanisation and the subsequent commercialisation of traditional health care systems coupled with cultural, social, and economic factors (Williams et al., 2000). The trade of medicinal plants has led to growing pressure on medicinal pant flora due to increased commercial collection, uncontrolled trade, and habitat loss (Barata et al., 2016; Chen et al., 2016).

In South Africa, several studies have shown that the medicinal plants' trade is dominated by Gauteng (Williams et al., 2000; 2001; 2007), KwaZulu Natal (Mander, 1998), Eastern Cape (Dold & Cocks, 2002), Limpopo (Tshisikhawe, 2002; Botha et al., 2004; Rasethe et al., 2019) and Mpumalanga (Botha et al., 2004). While the Free State province contributes little in terms of medicinal plant exports, little is known about the region's domestic trade (DAFF, 2016). Medicinal plant trade studies demonstrate

the significance of herbal medicine among its inhabitants, their concern about health as well as the socio-economic role played by the trade.

The increased demand for medicinal plants has made their use a public health problem due to the absence of quality and safety monitoring on the raw materials and products. As such, this predisposes the consumer to the risk of consuming mycotoxin-contaminated medicinal plants. Therefore, contamination of medicinal plants with mycotoxins presents an exceptional public health hazard due to associated mycotoxin adverse health effects. This review discusses the medicinal plant trade, preservation and storage methods, methods, and techniques for identifying fungi species and quantifying mycotoxins, important mycotoxins and their occurrences in medicinal plants and their products.

2.4 Medicinal plant supply chain

The increased volume of medicinal plants collected from the natural environment is attributed to the transition from conventional use to commercial trade (Van Wyk et al., 2018). The supply chain describes the entire process from sourcing medicinal plant materials from the suppliers/collectors/gatherers to the consumers (Mentzer et al., 2001). Sourcing /collection includes harvesting either whole plants or plant parts such as roots, bark, flowers, leaves, stems, and bulbs (Van Wyk et al., 2018). Previously, medicinal plant material harvesting was restricted to traditional health practitioners, but this landscape has changed (Van Andel & Havinga, 2008). To date, harvesting now involves commercial gatherers from urban and rural areas due to poverty and a high unemployment rate (Williams et al., 2000; Mander et al., 2007). Commercial gatherers' involvement means that people can access these medicinal plants far much easier without going to the traditional healers. Whilst this is quite good in terms of accessibility and affordability, it also puts pressure on the existing natural resources. It introduces safety and quality issues as traders or gatherers try to meet market demands.

The typical medicinal plant supply chain in South Africa involves four players: harvesters, wholesalers, retail markets, and consumers, as illustrated in **Figure 2.1,** except for pharmaceutical companies since they do not sell unprocessed medicinal plant material directly to the public. Harvesters collect medicinal plants from the wild and sell them at the markets to wholesalers, *muthi* shops, or street vendors (Mander

et al., 2007). In South Africa, for commercial purposes, the collection of medicinal plants, including protected species from the wild, is controlled, and a license is required to collect them (National Environmental Management: Biodiversity Act, 2004, Act No 10 of 2004). The trade of medicinal plants from Mozambique and Swaziland has been documented in the Durban and Johannesburg Street markets (Botha et al., 2004; Williams et al., 2007).

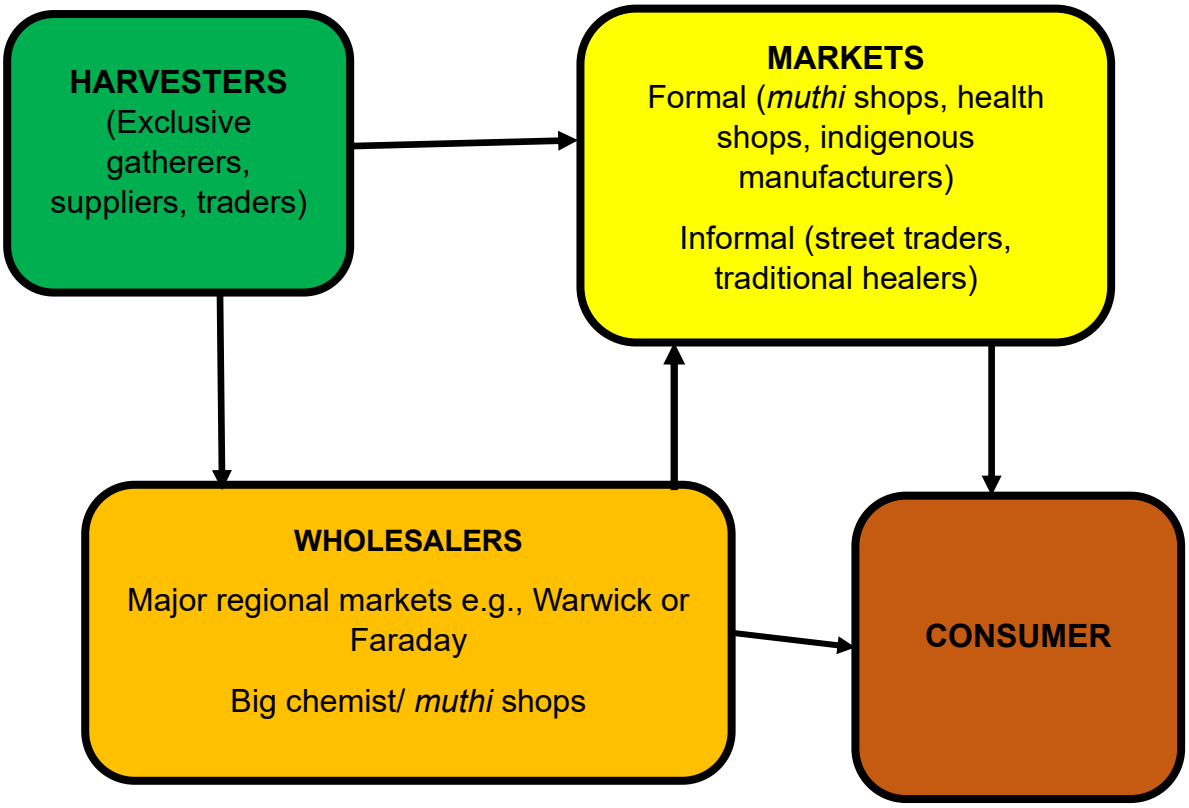


Figure 2.1: Medicinal plant supply chain (Adapted from Mander et al., 2007).

Wholesalers are aggregated bulk traders (a mixture of traders, harvesters, and street traders) from different ethnicities and provinces who sell other plants at a bigger permanent market (Williams et al., 2000). The medicinal plant retail trade is mainly composed of street traders and traditional healers (informal), *muthi* shops and herbal shops (formal) (Dold & Cocks, 2002; Mander et al., 2007). The medicinal plant trade mainly happens informally on street pavements, near taxi ranks, or at temporary pension day markets (Mander & Le Brenton, 2006; Ndhlala et al., 2011; Van Vuuren et al., 2014).

The major medicinal plant markets in South Africa include markets found in Durban (Warwick Triangle and Ezimbuzini), Johannesburg (KwaMai-Mai and Faraday markets), Limpopo province (Ga -Maja), and Pretoria (Marabastad) (Williams, 2003; Mander et al., 2007; Van Vuuren et al., 2014; Matoka & Masoko, 2017). Medicinal plant trade has also been reported in the Eastern Cape, Northern Cape, Western Cape, and Mpumalanga, whilst the Free State and Northwest provinces are the least studied (Dold & Cocks, 2002; Botha et al., 2004; Loundou, 2008; Philander et al., 2014).

Due to the complexity of the supply chain, it is challenging to monitor traded species' compliance or trace origins in the markets, causing the overexploitation of indigenous medicinal plant resources (Cunningham, 1993). Studies must focus on the least studied areas to adequately describe the medicinal plant trade in South Africa and assess species that should be promoted for commercial trade and those that influence sustainability initiatives. Furthermore, the Free State Province is in the middle of the country and has a unique supply chain rooted in Basotho traditional medicine. Market surveys have focused on the big permanent markets neglecting the role played by other scattered urban markets across South Africa. Considering the increased commercial trade in medicinal plants, current studies should also focus on small formal and informal markets to clearly describe the medicinal plant trade landscape in South Africa.

2.5 Preservation and storage

The growing demand for herbal and medicinal products shows that a high consumption market is developing, which requires raw materials of high quality (Van Wyk et al., 2018). In the production chain, medicinal plants' post-harvest cycle is critical as it affects the quality and amount of active ingredients in the finished product (Street et al., 2008; Rocha et al., 2011). The advancement of preservation techniques such as vacuum packing, drying, and freeze-drying, irradiation, pasteurisation, smoking, chemical additives, heating, cold refrigeration, freezing, dehydration, concentrating, microwave heating, salting, or pickling; and fermentation have contributed to the increased shelf-life of plant and animal foods over the past centuries (Augustin et al., 2016; Vaclavick et al., 2021). Drying is the most widely used method

for preserving medicinal plant materials using different techniques (Rocha et al., 2011). This review focuses only on the most utilised drying techniques.

2.5.1 Preservation - Drying

Drying using different methods has been practised since ancient times to preserve food from plant and animal origins, including herbs (Mohammad et al., 2019). The processing of herbs after harvest involves inspection and sorting, primary processing and drying (WHO, 2018). Preservation refers to processing done, or any form of treatment applied to plant material to prolong the shelf life of the raw material to last longer than its natural capability (Mohammad et al., 2019; Thamkaew et al., 2021). Medicinal herbs are dried immediately or shortly after harvesting to mitigate microbial contamination and loss of therapeutic ingredients unless they are meant for use in fresh form (WHO, 2018). Drying is ideal for preventing phytochemical alterations and ensuring long-term storage. Additionally, drying helps reduce shipment weight and transportation and storage costs (Rocha et al., 2011; Knorr & Augustine, 2021).

Water is an essential biological component that determines the medicinal plant's physical and chemical properties (Rocha et al., 2011). Plant parts, namely flowers, leaves, stems, or roots, contain relatively high moisture content between 5.0 and 9.0 kg/kg db (Martynenko and Kudra, 2015). In most situations, after harvesting, the fundamental step is to remove water by drying, which is the most common method to preserve medicinal plants (Muller & Heindl, 2006; Pitt & Antonello, 2016; Thamkaew et al., 2021). Drying mainly focuses on prolonging the storage life by reducing the water content to a level that inhibits microbial and enzymatic activity; prevents tissue deterioration and phytochemical alteration (Fennell et al., 2004; Duc Pham et al., 2019). Ideal drying conditions include a balance between temperature, air velocity, humidity, and air quality to facilitate the quick reduction in the plant materials' moisture content without significantly affecting the quality and therapeutic properties (Amit et al., 2017; WHO, 2018). Drying is necessary for grinding and milling to transform the materials into a convenient form with reduced weight and volume. Furthermore, it contributes to a consistent supply chain, facilitates plant marketing, and improves transport and storage (Karam et al., 2016; Amit et al., 2017; WHO, 2018).

Various drying methods depend on the plant type, environmental, economic factors, and desired quality aspects of the final product. The most critical elements for

medicinal plants after harvest that may alter the product's final quality are chemical changes, including changes in appearance (colour) and smell (Fennell et al., 2004; WHO, 2018; Thamkaew, 2021). Collectors, growers, and medicinal plant traders often overlook proper drying conditions and storage (Mander et al., 1998; Street et al., 2008; Van Vuuren et al., 2014). Despite technological advancement, a fundamental economic and ecologically important criterion in drying medicinal plants is determining the optimum conditions for different plant materials (Mullen & Heindl, 2006; Amit et al., 2017; Duc Pham et al., 2017). Therefore, the choice of an appropriate method is crucial. The common drying methods used in drying medicinal herbs include natural and artificial drying (WHO, 2018).

2.5.1.1 Natural drying

Natural drying is the easiest and most common method of drying medicinal plants in Africa and other tropical and sub-tropical regions which experience hot and dry weather conditions (Muller & Heindl, 2006; WHO, 2018; Thamkaew et al., 2021). Typically, natural drying is usually done for manually collected plants in a well-ventilated area with direct (sun drying) or no direct sunlight (shade drying) and is commonly used where the plants mature and are harvested in the dry season (Oztekin & Martinov, 2014; WHO, 2018).

The exposure to the sun's rays or the dehydrating effect of air streams accelerates moisture removal from the material. Sun-drying causes a considerable change of colour and aroma change in the dried herbs (Thamkaew et al., 2021). Shade drying utilises solar energy as a heating source. The process is similar to sun drying, except that the herbal materials are not exposed to direct sunlight but are placed in a shaded area with adequate ventilation and low humidity. Shade drying has an advantage over sun drying as it allows the preservation of photo-sensitive phytochemicals and minimises oxidation due to light-induced chemical reactions (WHO, 2018; Thamkaew et al., 2021). Although shade drying is slow, it is most preferred when it is necessary to maintain the natural appearance and taste of leaves and flowers. Both sun and shade drying are widely used in rural and urban areas by both people and small businesses due to the low cost but providing good quality dried products (Thamkaew et al., 2021).

2.5.1.2 Artificial heat / Hot air drying

Drying using artificial heat, also known as convective drying, is commonly used in non-tropical countries where solar energy is inadequate for sun and shade drying. It is quicker than open-air drying and often is necessary during rainy days or in high-humidity areas (WHO, 2018). Artificial heat systems use hydrocarbon fuels or electricity to heat incoming air to facilitate the evaporation of moisture and volatile compounds (Orphanides et al., 2016, WHO, 2018). The main advantage of hot air drying is allowing users to control drying parameters such as time, air velocity and temperature to achieve the desired product quality (Thamkaew et al., 2021).

The temperature, humidity, and other conditions for artificial heat-drying should be determined by the physical nature of the dried herbal content and physical/chemical properties (WHO, 2018). High temperatures can cause excessive loss of volatile components or chemical ingredients degradation. A study by Roshanak et al. (2016) reported that general drying increased antioxidant activity, TPC, TFC and chlorophyll content, while it decreased vitamin C.

An earlier study also showed that the moisture loss (%) from the four medicinal species was the highest following the oven drying method. The moisture loss (%) was almost the same in the case of solar and natural drying methods. In contrast, oven drying resulted in the lowest essential oil content compared with the other methods. Regarding the colour of the products, i.e., their chlorophyll contents were higher due to solar or natural drying than in the case of oven drying (Kassesm et al., 2006). Generally, bark and root material drying should be below 60 °C and 40 °C for leaves, herbs, and flowers (Oztekin & Martinov, 2014; WHO, 2018). However, the main limitations of hot air drying include high energy costs and shrinkage of the products (Orphanides et al., 2016).

2.5.2 Storage

Herbal raw materials and their products should be preserved under suitable hygienic, temperature and relative humidity conditions for long-term storage and future use. The purpose of preserving and storing medicinal plants is to avoid deterioration of their

quality and microbiological contamination (WHO, 2018). Poor storage conditions and methods can result in physical, chemical, and microbial changes, which have health and economic impacts (Kent et al., 2018). In Brazil, medicinal plants' recommended storage period is one year (Carvalho et al., 2014). Nevertheless, a more extended validity period may be acceptable, subject to the manufacturer presenting stability test results that show preservation of the product's characteristics (Carvalho et al., 2014). Therefore, storage should consider appropriate and suitable packaging that best protects the processed material from physical damage whilst preventing exposure to adverse conditions and maintaining the stored material's physical and chemical characteristics (Kent et al., 2018; WHO, 2018).

A study by Mander et al. (1997) found that medicinal plant collectors usually build up stocks in the countryside before an order can be made or before enough material is available to warrant a trip to the market. Raw materials for wholesale trading, in contrast to those for retail, such as whole plants, plant parts and chopped material, are stored in recycled plastic woven bags (50 kg capacity) (Mander et al., 1997). This form of storage is most prevalent and primarily utilised across the medicinal plant supply chain. The bags are usually stored in dry areas, preferably under the shade, to prevent plant material from decomposing, physical deterioration, and contamination. Whereas the rooms of shops and healers typically have an area where large numbers of these bags are stored (Mander et al., 1997).

Depending on the nature of the business, various containers are used to store retail materials for direct sales to consumers. Street traders store their bagged goods under plastic sheets in the street at the end of every working day (Mander, 1998). In shops, products are usually stored on shelves or in 'pigeonholes' where the vast array of plant material can be kept relatively neat and separately stored (Mander et al., 1997). Room temperature and humidity conditions must be controlled during storage to help prevent the stored materials from deteriorating due to metabolic activity (Silva et al., 2013). Furthermore, storage areas should ensure correct stocking, allowing for proper separation and segregation of raw, unprocessed and processed herbal materials. Monitoring and control systems and accurate record-keeping should also be used where appropriate to ensure good storage conditions and to comply with the "first-in and first-out" principle (WHO, 2018).

Mbendana et al. (2019) reported different storage practices for processed and unprocessed herbal products at KwaMai-Mai and Marabastad *muthi* markets. Herbal items were placed in direct air racks, while others were stored in re-used containers such as soft drink bottles, aluminium cans, milk containers, peanut butter jars and newspapers. Samples of raw medicinal plants were stored at room temperature for a year, and after microbiological evaluation, it was found that most of the samples had bacterial and fungal species (Chandra et al., 2019).

Few studies have focused on the effect of harvest and duration of storage on the quality and efficacy of medicinal plants (Fennell et al., 2004; Laher et al., 2013). As mentioned, collectors harvest medicinal plants from the wild resources and considering the post-harvest factors during preservation and storage; these plant materials are susceptible to fungal and mycotoxin contamination post-harvest.

2.6 Fungal contamination

The role played by fungi in deteriorating stored herbal drugs and reducing their medicinal potential has been a global concern for decades (Halt, 1998; Altyn & Twaruzek, 2020). Fungi species are among the largest organisms on earth, with at least -5.1 million to a trillion species globally, but only 100 000 have been named (Blackwell, 2011; Hawksworth & Lucking, 2018). Fungi are plant-like with no chlorophyll or heterotrophic saprophytes in nature, deriving their foods from other organisms and the surrounding environment. Over 300 species are known to exert adverse effects on humans and animals by producing mycotoxins, and over 100 species have been implicated in food spoilage (Pereira et al., 2014; Alshannaq & Yu, 2017). Major fungi genera which are toxigenic include but are not limited to species belonging to *Aspergillus, Fusarium, and Penicillium*. Species of *Aspergillus* and *Penicillium* are either commensal or pathogenic and responsible for post-harvest contamination and can produce toxins during drying and storage. *Fusarium* species contaminate plant materials before and during harvesting (Pitt, 2000; Zain, 2011; IARC, 2012).

2.6.1 Major fungal contaminants

2.6.1.1 Aspergillus

The *Aspergillus* genus belongs to the Ascomycota phylum, Eurotiales order and Trichocomaceae family. Over 180 species of this genus have been recognised, while 30 are well defined and can be easily identified (Pitt, 2000). The *Aspergillus* species are among nature's most abundant and widely distributed filamentous saprophytes and the most economically significant fungal genera (Klich, 2002). They are hyphomycetes characterised by their distinctive conidiophore with large, heavy-walled stripes that consist of a swollen apex termed vesicle (Pitt & Hocking, 1997; Klich, 2002). Twenty *Aspergillus* species are human and animal pathogens (Pitt, 2000). *A. flavus* and *A. parasiticus* mainly occur in warmer climates and are the most important primary producers of aflatoxins (AF). *Aspergillus* species have been reported to produce various mycotoxins not limited to aflatoxins, fumonisins (FBs), ochratoxins (OT) and patulin (PAT) (IARC, 2012). Other important species include *A. clavatus, A. fumigatus, A. terreus, A. versicolor, A. wentii* and *A. cryzae* (Klich, 2002).

2.6.1.2 Fusarium

Fusarium species belong to the Ascomycota phylum, Hypocreales order and the Nectriaceae family. Nine to sixty species have been recognised under this genus, and thirty have been identified (Pitt & Hocking, 1997). Micromorphological characterisation shows that *Fusarium* species have uncoloured large (25 µm to 50 µm) multiseptated, and curved conidia called macroconidia which are unique for *fusarium* (Leslie & Summerell, 2008). *Fusarium* species are common in the soil and produce a range of different toxins, including trichothecenes such as deoxynivalenol (DON), nivalenol (NIV) and T-2 and HT-2 toxins, as well as zearalenone (ZEN) and fumonisins (IARC, 2012). The most isolated species are *F. verticillioides* and *F. graminearum. F. verticillioides* is an abundant plant endophyte producing fumonisins in drought-stressed or insect-damaged plants. *F. graminearum* is the primary producer of DON and ZEN. Other species of the genus *Fusarium* include *F. acuminatum, F. avenaceum, F. culmorum, F. chlamydosporum, F. oxysporum, F. proliferatum, F. solani, F. sporotrichiodes,* and *F. subglutinans* among others (Leslie & Summerell, 2008; Munkvold, 2016).

2.6.1.3 Penicillium

Penicillium belongs to *the* Ascomycota phylum, Eurotiales order, and Trichomaceae family. *Penicillium* is a large genus with over 354 accepted species that have been recognised, with over 50 species commonly occurring (Visagie et al., 2014). They are filamentous saprophytic fungi, and their phialides are grouped singly or in groups in a finger-like cluster, a structure termed penicillus (Pitt & Hocking, 2002; Visagie, 2014). *Penicillium* species can grow in any environment with some mineral salts but slowly and have green macroconidia. *Penicillium* species have been reported to produce several mycotoxins under certain conditions, namely citrinin, ochratoxins, patulin, and other tremorgenic toxins. Isolated *Penicillium* species in food include *P. citrinum, P. commune, P. chrysogenum, P. crustosum, P. cyclopium, P digitatum, P. frequentans, P. expansum, P. madriti, P polonicum, P. roqueforti, P. verrucosum,* and *P. viridicatum* among others *(*Pitt & Hocking, 1988, 2002, 2009; Visagie et al., 2014).

2.6.2 Factors influencing fungi contamination.

Many factors influence fungal colonisation or mycotoxins production, which can be broadly categorised into physical and biological factors. Physical factors include environmental conditions favourable to fungal colonisation, such as temperature, relative humidity, and insect damage (Zain, 2011; Tola et al., 2016; WHO, 2018). Biological factors involve the interaction between the colonising toxigenic fungal species (genotype) and substrates. The main factors influencing fungi growth and development include nutritional content of substrate, temperature, light, aeration, pH, and water activity are summarised below (Zain, 2011).

Water activity plays a significant role in the colonisation of a substrate by fungi. It enables fungi to digest complex compounds during primary and secondary metabolism and synthesise new compounds for growth (Muller et al., 2006). Whilst all moulds need moisture for development, the moisture requirements widely differ. Moulds that can grow in very low water activity are referred to as xerophiles. Xerophytic fungi can take water vapour out of the air for growth, whereas hydrophilic fungi can grow at a very high-water activity (Sautour et al., 2002; Gock et al., 2003;). Depending on the plant material's water activity, some fungi species may grow within

the substrate. As described in the preceding sections, drying is done immediately after harvesting to reduce the plant material's moisture content to mitigate fungal growth.

Regarding nutritional requirements, various mould species might prefer similar nutrients, or the requirements might vary. Some moulds may prefer simple sugars, others can utilise complex sugars, while others thrive on substrates with high salt content (Pitt & Hocking, 2009; Rico-Munoz et al., 2019). Fungal species can be classified into three groups based on their growth temperatures, namely thermophilic (<35 °C), mesophilic (5 -35 °C), and psychrophilic (<5 °C). Psychotolerant species can grow at 0 °C and standard room temperature (Pitt & Hocking, 2009; Passamani et al., 2014). The growth of mould species also depends on light availability; some mould species prefer dark or daylight. Whilst others prefer alternate light and darkness for sporulation. Furthermore, most moulds require air for growth but with different pH requirements. Most of them can grow over a pH range of 3 -7 (Gock et al., 2003; Rico-Munoz et al., 2019).

2.6.3 Fungal contamination in South African medicinal plants

The contamination of medicinal plants by toxigenic or non-toxigenic fungi has been documented in South Africa. The presence of fungi presents a risk of mycotoxin occurrence in these plant materials, loss in quality and appearance, and stock loss. **Table 2.1** shows the commonly isolated fungal species documented in the South Africa for the past twenty years (2000 - 2020).

Table 2.1: Fungal contamination in South African medicinal plants

Location	Fungal Genus	References
Eastern Cape (Nelson Mandela Bay)	*Aspergillus, Mucor, Penicillium*	Sewram et al, 2006; Govender et al., 2006
Western Cape (Cape Town) and Gauteng (Tshwane)	*Aspergillus, Fusarium, Penicillium*	Katerere, 2008
Gauteng (Mai Mai Market Johannesburg)	*Alternaria, Aspergillus, Cladosporium, Fusarium, Penicillium, Rhizomucor, Rhizopus*	Sithole et al., 2017

As illustrated in **Table 2.1,** relatively few published studies have been conducted on the fungal contamination of medicinal plants despite the growth and extensive use of medicinal plants in South Africa. As mentioned, formal and informal trade is conducted in open markets or shops where physical and biological factors that promote fungal contamination exist. Therefore, this warrants more studies to determine mycoflora prevalence in marketed medicinal plants to ensure their quality and safety.

2.6.4 Effects of fungal contamination

Fungal species can be pathogenic to plants and animals, including humans, cause food spoilage, or produce toxins depending on physical and biological factors (Pitt, 2000, 2009; Rico-Munoz et al., 2019). The fungal species responsible for the spoilage of foods tend to differ according to the food product's properties, the physiochemical properties and the processing methods used. Usually, the adulteration of processed foods commences with physical contamination by food spoilage organisms, followed by subsequent multiplication of these undesirable microorganisms (Rawat, 2015). Fungi contamination may lead to the degradation of products, resulting in significant economic losses and risks to consumer health when mycotoxin-producing fungi are involved. Fungi can also colonise plants in the field and produce mycotoxins which may continue during storage. Mycotoxins produced by toxigenic fungi cause numerous diseases termed mycotoxicosis, among other harmful effects (Pitt, 2000; Zain, 2011; Alshannaq & Yu, 2017).

Human oesophageal cancer is associated with ingesting corn contaminated with *Fusarium verticillioides* (Rheeder et al., 1992; Marasas, 2001). Some *Aspergillus* species are pathogenic; for example, *Aspergillus fumigatus,* which is an opportunistic fungus that causes Aspergillosis in humans (Paulussen et al., 2017). Aspergillosis occurs in the form of chronic pulmonary aspergillosis (CPA), aspergilloma or allergic bronchopulmonary Aspergillosis (ABPA). Other non-invasive manifestations of Aspergillosis include fungal sinusitis, otomycosis, keratitis and onychomycosis (Shetty et al., 1997; McCormick et al., 2010; Paulussen et al., 2017). The estimated worldwide prevalence of significant recurrent, aggressive, and inflammatory Aspergillosis triggers about 600 000 deaths (Denning et al., 2013). In animals, particularly chicken, *Aspergillus* spp. causes brooder disease – a common pneumonia of the lungs (Arné et al., 2011). While human *Penicillium* pathogenic species are rare, opportunistic infections due to *Penicillium marneffei* have been reported (Vanittanakom et al., 2006). The presence of microbial contaminants in medicinal plants can compromise the therapeutic efficacy of the medicinal plants or their products and adversely affect the health of patients taking the medicinal plants as medication.

2.7 Fungi Identification

Accurate and precise identification and differentiation of fungal species is important but challenging (Raja et al., 2017). Various methods have been used to identify and differentiate fungi species broadly falling under morphology, microscopy and molecular techniques, as illustrated in **Table 2.2.**

Table 2.2: Fungal species identification methods

Matrix	Fungal Genus	Techniques	References
Indian medicinal herbs and spices	*Aspergillus, Penicillium, Mucor, Cladosporium, Fusarium,Alternaria and Rhizopus.*	Morphology	Aiko and Mehta, 2016
Marketed medicinal plants in Brazil	*Cladosporium, Fusarium, Aspergillus* and *Penicillium.*	Morphology (identification based on fungal keys.	Pereira et al., 2015
Chinese medicinal herbs	*Aspergillus, Penicillium, Eurotium* and *Cladosporium.*	Morphology & Molecular	Zheng et al., 2017
Kenyan market medicinal herbal products	*Aspergillus, Penicillium, Saccharomyces, Rhizopus, Rhodotorula, Cryptococcus, Basidiobolus, Mucor, Malbranchea, Absidia, Trichophyton, Scedosporium, Fusarium, Candida, and Saccharomyces.*	Morphology & Microscopy	Keter et al., 2017
Chinese root herb	*Aspergillus* and *Penicillium.*	Morphology & Molecular	Su et al., 2018
Medicinal and edible Cassiae Semen	*Aspergillus, Penicillium, Candida,* and *Schizophyllum*.	Molecular	Guo et al., 2020
Myristicae Semen (nutmeg)	*Aspergillus, Xerochrysium, Xeromyc, Hyphopichia, Alternaria, Chaetomium, Trichothecium,* and *Penicillium.*	Molecular	Jian et al., 2020
Kingdom of Saudi Arabia. Agarwood	*Aspergillus, Penicillium, Fusarium* and *Rhizopus*	Morphological & Molecular	Al-Hindi et al., 2020

Morphological identification by culturing has limited value in identifying and comparing moulds to species level. Natural variations between isolates and variations of colonies on various culture media make culturing alone inadequate but can supplement microscopic examinations (Ko et al., 2011; Vesper, 2011). Microscopy is useful for evaluating fungal species morphology due to minimal microscopic characteristics variations. Fungal identification keys are critical for accurate and proper identification using microscopy, offering standardisation using specific media (Vesper et al., 2011; Vyzantiadis et al., 2012). Molecular techniques utilising newly emerging high-performance techniques, such as next-generation sequencing (NGS) or proteomics, provide new pathways for more adaptive, reliable, precise, and accelerated diagnosis of fungal species identification and differentiation (Gabaldon, 2019).

Other mass spectrometry-based methods such as Matrix-Assisted Laser Desorption Ionization Time-Of-Flight (MALDI-TOF), Mass Spectrometry, Fourier Transform Infrared (FT-IR) spectroscopy and Raman spectroscopy also hold considerable promise for the identification of fungi in the future (Vyzantiadis et al., 2012; Gabaldon, 2019). Where possible, the identification of fungi species should use a combination of micromorphological, cultural and molecular characteristics (Raja et al., 2017). Nevertheless, there has been a gradual shift from the traditional fungi identification methods based on morphology to new molecular-based techniques which offer better precision and high throughput.

2.8 Microbial limits (Pharmacopeia Regulations)

Through pharmacopoeias, various regulations establishing high-quality standards for medicinal plants and related products are shared internationally, while legal frameworks exist at national or regional levels (WHO, 2016). The World Health Organisation reports that over 100 countries worldwide have regulations for herbal medicinal products, which differ significantly in many cases.

The pharmacopoeia microbial limits do not distinguish between mould species as they do for bacterial species. It gives the total combined mould and yeast count. The international pharmacopoeia (WHO, 2016) states the limit for plant materials for

internal use at 10^2 colony forming units (CFU). United States Pharmacopeia describes botanical ingredients and products at 10^5 CFU. Whilst European pharmacopoeia puts the limit for herbal medicinal products at 10^4 CFU. Regulations are essential as inferior quality, counterfeit or adulterated herbal products seriously threaten consumer safety globally (WHO, 2013). The African Herbal Pharmacopeia published by the Association of African Medicinal Plant Standards in 2010 does not provide for microbial limits in medicinal plants and their products (AAM, 2010). National pharmacopoeias have been published in several African countries, including Ghana, Nigeria, and Madagascar.

South Africa does not have a published Pharmacopeia, but it is an observer member of EU Pharmacopeia (Brennan, 2013). In South Africa, the microbiological quality of complementary medicines, including herbal medicines, is controlled by the Medicines Control Council, which recognises other Pharmacopeia such as British Pharmacopeia and United States Pharmacopeia (Brennan, 2013). The absence of specific limits for moulds and yeasts in medicinal plants indicates that no quality monitoring is conducted. Countries are not prioritising monitoring medicinal plants' contamination with fungi as they do for other bacterial contaminants. The absence brings out the need for more studies to determine fungi incidence and occurrences in medicinal plants to influence the development of standards of limits for mycobiota in medicinal plants and their products.

2.9 Mycotoxins

Mycotoxins are toxic compounds produced naturally during the secondary metabolism of certain types of filamentous fungal species (Bennett, 1987; Zain, 2011). They are low molecular weight compounds which range from basic C_4 compounds such as moniliform to complex substances such as phomopsins produced by *Phomopsis* species (Dinis et al., 2007; Zain, 2011). Although fungi produce all mycotoxins, not all toxic compounds produced by fungi are referred to as mycotoxins (Bennet & Klich, 2003). Low molecular weight metabolites (MW~ 700 Daltons) are only toxic at high concentrations and are not considered mycotoxins, such as ethanol. Mycotoxins do not exert any biochemical effects on fungal growth and development (Bennett, 1987; Dinis et al., 2007).

2.9.1 Factors contributing to mycotoxin production.

Numerous biological, physical, and chemical factors influence mycotoxin contamination in food and plant materials. Moulds may grow on plants during pre-harvest or post-harvest periods; henceforth, raw plant materials and their products can be contaminated with mycotoxins at various supply chain stages (Bennett, 2003, Luo et al., 2018; WHO, 2018). The key physical determinants of mycotoxin production pre- and post-harvest are relative humidity, temperature, and insect infestation (Zain, 2011; Agriopoulou et al., 2020). Also, drought stress can selectively influence the colonisation and metabolism of mycotoxigenic fungi and therefore, alter mycotoxins production (Zain, 2011; Perrone et al., 2020). Biological factors involve the interaction between the colonising toxigenic fungal species and substrate. Biological factors are sub-categorised into inherent variables, including fungal types, strain mutability, strain heterogeneousness, and instability of toxigenic products (Tola et al., 2016; Perrone et al., 2020). In comparison, chemical factors include carbon dioxide and oxygen air concentrations, mineral nutrition, and prior treatments such as the use of fungicides or fertilisers (Savi et al., 2013; Agriopoulou et al., 2020).

2.9.2 Major mycotoxins

More than 400 mycotoxins have been identified, but research attention has focused primarily on those that are carcinogenic and/or toxic. The most critical agro-economic mycotoxins are aflatoxins, fumonisins, ochratoxins, trichothecenes and zearalenone (Alshannaq & Yu, 2017; Agriopoulou et al., 2020). This review focuses on the most frequently reported mycotoxins in medicinal plants and their products, namely aflatoxins (AFs), fumonisins (FBs), ochratoxins (OTA), deoxynivalenol (DON), toxins (T-2 & H-2), zearalenone (ZEN).

2.9.2.1 Aflatoxins (AFs)

Aflatoxins are difuranocoumarin derivatives produced by the polyketide pathway by members of the fungal genus *Aspergillus,* which contains approximately 250 known species (Klich, 2007; Smith & Groopman, 2018). *Aspergillus flavus* and *Aspergillus*

parasiticus are the major aflatoxin producers, with other species being encountered less often (Klich, 2007; Peng et al., 2018).

There are more than twenty known aflatoxins, but the six main types of aflatoxins are common; namely, AFB_1, AFB_2, AFG_1, AFG_2, AFM_1 and AFM_2 (illustrated in **Figure 2.2**) and are important to humans, animals, agri-food, and feeds. AFM_1 is a metabolic product of AFB_1 found primarily in animal tissues and fluids and mainly detected in milk and urine. It does not contaminate food or feed but is due to ingestion of feed contaminated with AFB_1 (Smith & Groopman, 2018). AFB_1 is the most potent aflatoxin and is classified as Group 1 carcinogen together with AFB_2, AFG_1, and AFG_2 (IARC, 2002; Ostry et al., 2017).

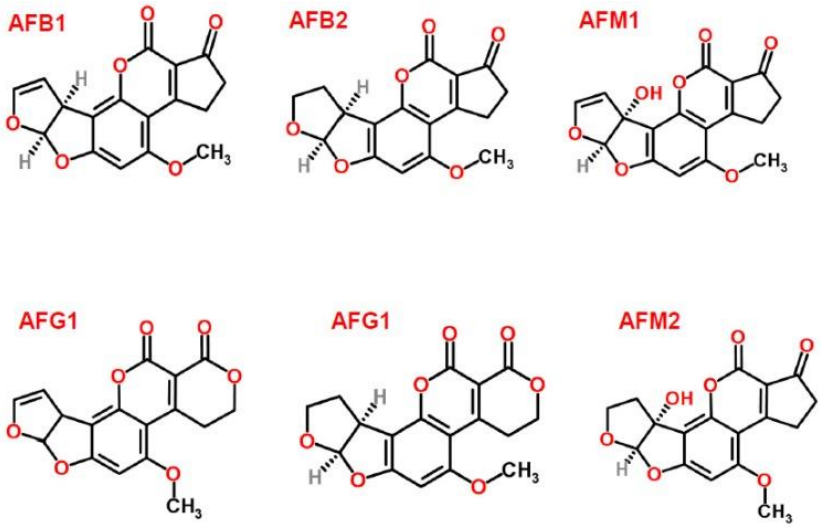


Figure 2.2: Chemical structures of aflatoxins (Alshannaq & Yu, 2017).

2.9.2.2 Deoxynivalenol (Vomitoxin)

Deoxynivalenol (DON), also known as vomitoxin, is a type B trichothecene. It belongs to an epoxy-sesquiterpenoid mycotoxin, mainly produced by *Fusarium* species (*F. graminearum* and *F. culmorum)* (Richard, 2007; Pestka & James, 2010). The acetylated DON derivatives are 3-acetyl-deoxynivalenol (3-ADON), 15-acetyl-deoxynivalenol (15-ADON), the plant-derived DON-glucoside (DON 3G), as illustrated in **Figure 2.3** and the bacterial product de-epoxy-DON (DOM-1) (Pestka, 2010). DON

is formed in the field before harvesting and is very difficult to control due to weather conditions. It also causes adverse health effects in both animals and humans, including swine feed rejection and vomiting. DON causes dermatosis, gastrointestinal haemorrhaging, and inhibition of DNA and protein synthesis (Agriopoulou et al., 2020; Pestka, 2010). It is also a neurotoxin and immune depressant and has been categorised as a Group 3 carcinogen (Ostry et al., 2017).

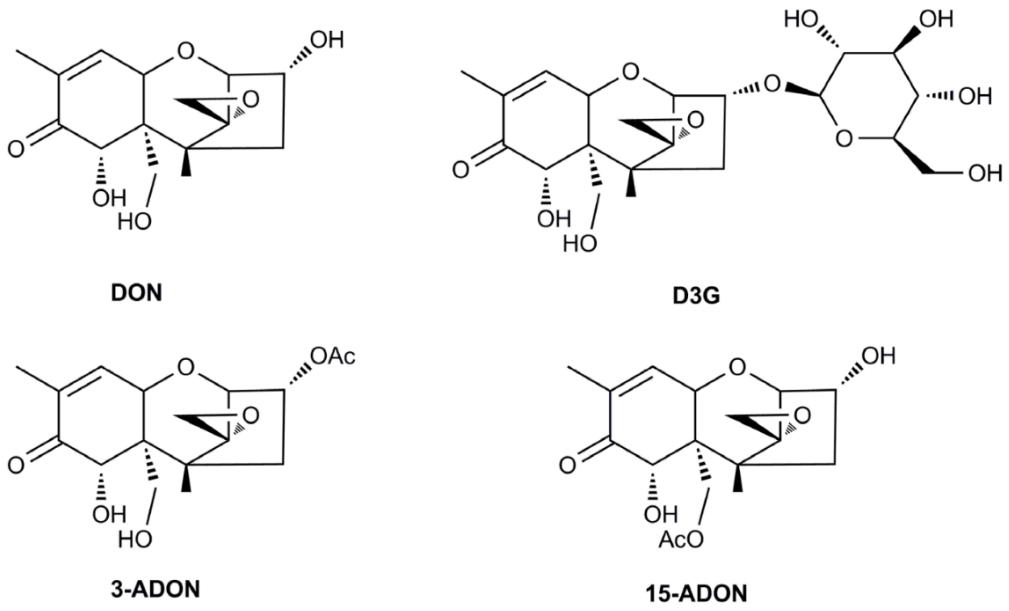


Figure 2.3: Chemical structure of Deoxynivalenol and its derivatives (Tian et al., 2016).

2.9.2.3 Fumonisins (FBs)

Fumonisins are commonly referred to as *Fusarium* toxins since they are mainly produced by *Fusarium* species. Various fungi species with different toxin-producing abilities produce fumonisins. *Fusarium verticillioides* and *Fusarium proliferatum* are the primary producers. Other species include *F. anthophilum, F. bulbicola, F. fujikuroi, F. globosum, F. nygamai, F. oxysporum, F. phyllophilum, F. ramigenum,* and *Aspergillus niger*. Fumonisins are a group of non-fluorescent polyketide mycotoxins (O'Donnell et al., 2018; Pereira et al., 2019). Compared to other mycotoxins, fumonisins' unique physical properties include solubility in acetonitrile, methanol and water, thermal stability, resistance to alkalis and no sensitivity to light (Jackson & Jablonski, 2004). Currently, 28 fumonisins have been isolated and divided into four groups, A, B, C and

P, wherein the ‘A’ series are amides, and the ‘B’ series possesses a free amine, as illustrated in **Figure 2.4** (Rheeder et al., 2002; Braun & Wink, 2018). The most widespread naturally occurring fumonisins are fumonisin B derivatives, which include FB_1, FB_2 and FB_3. They have been reported to induce hepatotoxicity, nephrotoxicity, and immunosuppressive activity in human oesophageal cancer (Alshannaq and Yu, 2018; Braun & Wink, 2018). Among these, FB_1 is the most common and toxic and has been classified as a group 2B human carcinogen (Ostry et al., 2017)

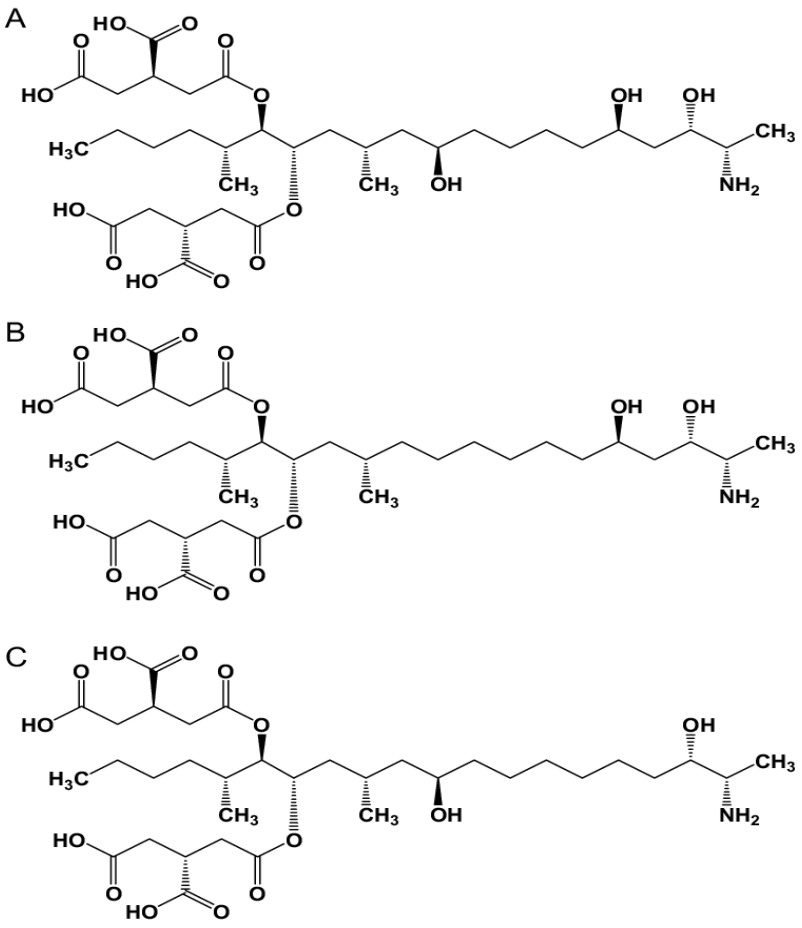


Figure 2.4: Chemical structures of Fumonisins (Kostić et al., 2019).

2.9.2.4 Nivalenol (NIV)

Nivalenol is a type B trichothecene, with the molecular structure illustrated in **Figure 2.5.** It is a mycotoxin produced by *Fusarium* species, namely *F. cerealis*, *F. culmorum F. crookwellense, F. episphaeria, F. graminearum, F. nivale* and. *F. poae*. The toxic

effects of nivalenol include bone marrow toxicity, erythropenia, leucopenia, haemorrhage, toxicity to lymphoid organs, gastrointestinal distress, and damage to epithelial membranes of the intestine, the thymus and testis (Pestka, 2010; Patriarca et al., 2017). NIV acute exposure causes emesis in susceptible species, while low-dose chronic exposures can cause growth retardation and immunotoxicity. Higher doses of NIV affect reproduction and development (Pestka, 2010). The overall conclusion was that the NIV carcinogenicity is not classifiable (group 3) (IARC, 1993; Ostry et al., 2017.

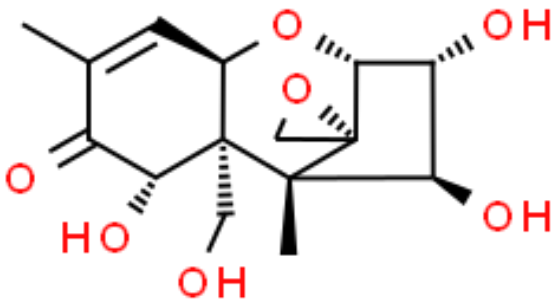
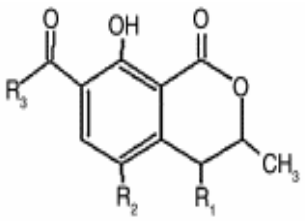


Figure 2.5: Nivalenol molecular structure (Royal Society of Chemistry, 2022)

2.9.2.5 Ochratoxin (OT)

Ochratoxin (OT) is a pentaketide mycotoxin produced by *Aspergillus* and *Penicillium* fungi species, mainly *A. carbonarius, A. niger, A. ochraceus* and *P. verrucosum*. A typical structure of OTA is shown in **Figure 2.6.** Ochratoxin comes in three secondary metabolite forms A, B, and C. Ochratoxin B (OTB) is a non-chlorinated form of Ochratoxin A (OTA), whilst Ochratoxin C (OTC) is an ethyl ester form of OTA, and ochratoxin α (OTα) has a phenylalanine moiety missing. Other OT-producing species include *A. melleus, A. sclerotiorum,* and *A. sulphurous* (Malir et al., 2016; Patriarca, 2017; Perrone et al., 2017). Among the three ochratoxins, OTA is the most toxic and widespread mycotoxin. OTA is reported to be nephrotoxic, hepatotoxic, teratogenic, and immunotoxic to several animal species and causes kidney and liver tumours in

mice and rats (Perrone et al., 2017; Liew et al., 2018; Wang et al., 2019). It has been classified as a possible human carcinogen (group 2B) (Ostry et al., 2017)



Ochratoxins	R1	R2	R3
OTA	H	Cl	-NH-CH(COOH)-CH_2- Phenyl
OTB	H	H	-NH-CH(COOH)-CH_2- Phenyl
OTC	H	Cl	-NH-CH($COOC_2H_5$)-CH_2-Phenyl
4-hydroxyochratoxin A	OH	Cl	-NH-CH(COOH)-C_2H- Phenyl
OTα	H	Cl	-OH

Figure 2.6: Ochratoxin chemical structure (Ringot et al., 2006).

2.9.2.6 T-2 and HT-2 Toxins

T-2 toxin (T2) and its deacetylated form HT-2 toxin are type A trichothecenes, which share a common tetracyclic ring system (**Figure 2.7**). HT-2 is a significant metabolite of T-2 and lacks an acetyl group at the C-4 position (Rocha et al., 2005). *Fusarium langsethiae* is the leading producer of T-2, while *F. equiseti, F. poae* and *F. sporotrichioides* are responsible for HT-2 mycotoxin production (Agriopoulou et al., 2020). Toxic effects of Alimentary Toxic Aleukia (ATA) due to human intoxication of T-2 and HT-2 include sepsis, growth retardation, necrotic lesions on contact sites, haemorrhages, myelotoxicity, inhibition of haematopoiesis and lymphoid depletion (Agriopoulou et al., 2020; Patriarca & Pinto, 2017). T-2 toxins are not classifiable as to their carcinogenicity to humans (Group 3) (Ostry et al., 2017). The chemical structure of T-2 and HT-2 toxins is illustrated in **Figure 2.7**.

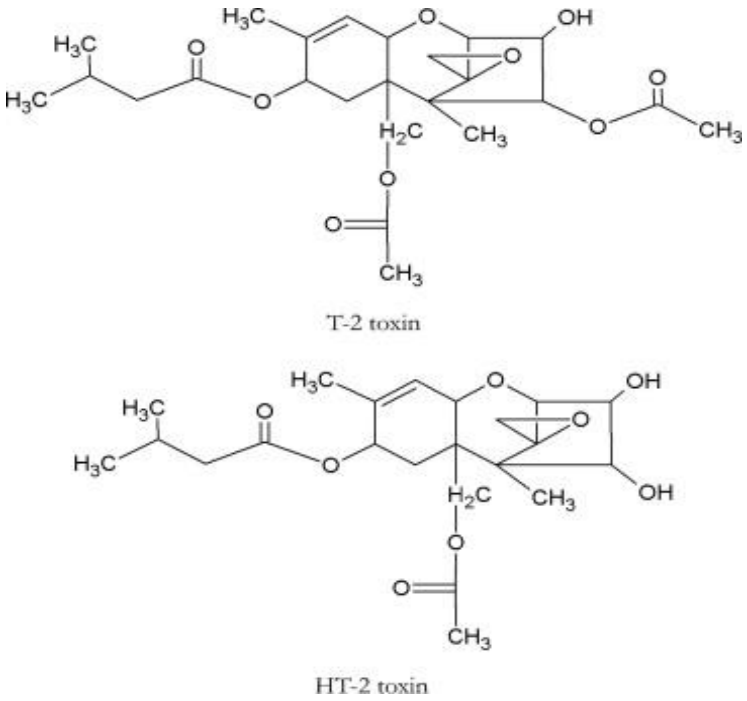


Figure 2.7: Chemical structure of T-2 and H-2 Toxins (Marin et al., 2013).

2.9.2.7 Zearalenone (ZEN)

Zearalenone or F-2 toxin is a phenolic resorcyclic acid with no steroid structure – secondary metabolite biosynthesised through a polyketide pathway by various *Fusarium* species. It is produced primarily by *Fusarium graminearum* and other *Fusarium* species, namely *F. culmorum, F. cerealis, F. crookwellense, F. equiseti, F. oxysporum, F. roseum, F. semitectum* and *F. sporotrichioides* (Bennett & Klich, 2003; Ashiq et al., 2014; Rogowska et al., 2019). The primary biologically active and reductive metabolites of ZEN in animals and humans are α & β -Zearalenol. ZEN (**Figure 2.8**) is a stable compound during storage/milling and food processing/cooking. It does not degrade at high temperatures and exhibits fluorescence under ultraviolet (UV) light (Alshannaq & Yu, 2017; Rogowska et al., 2019). Therefore, consumers can be exposed to ZEN directly by contaminated food or indirectly through products derived from animals exposed to mycotoxins. ZEN health effects in humans and animals include oesophageal cancer, reproductive disorders, hepatotoxic, haematotoxic, immunotoxic and genotoxic, and are classified as a Group 3 carcinogen (Ostry et al., 2017; Patriarca & Pinto, 2017; Yang et al., 2019).

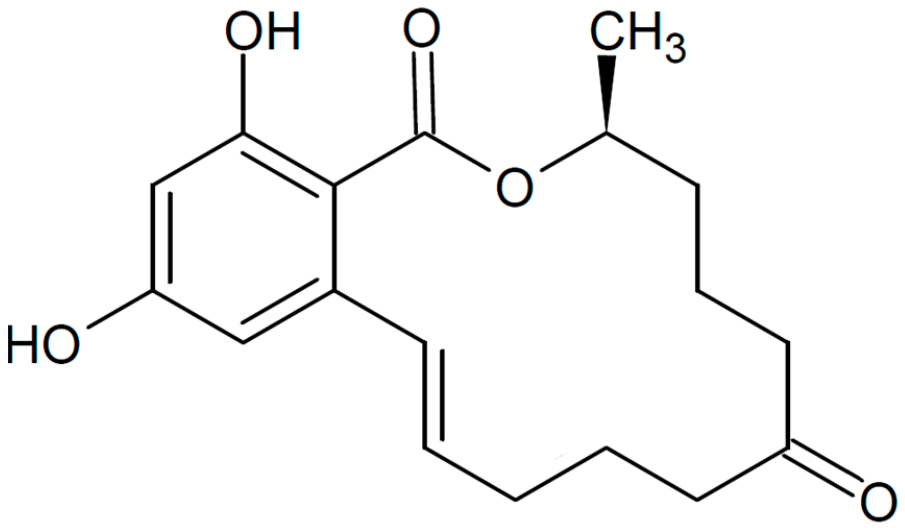


Figure 2.8: Chemical structures of Zearalenone (ZEN) (Ropjeko & Twaruzek, 2021).

2.10 Mycotoxin occurrence in African medicinal plants

Medicinal plants are prone to frequent contamination with toxigenic fungi pre-and post-harvest, with the fungi originating mainly from the soil (Ashiq et al., 2014). Mycotoxin occurrence has been reported in medicinal plants, their preparations, and herbal medicines from various countries. Mycotoxin contamination is more common in tropical and subtropical regions as high temperatures and moisture content contribute to fungal development and toxin production (Patriarca & Pinto, 2017; Agriopoulou et al., 2020). Mycotoxin contamination studies published between 2000-2020 are illustrated in **Table 2.3.** There are only a few studies on the simultaneous occurrence of mycotoxins in medicinal plants in South Africa (Sewram et al., 2006; Katerere et al., 2008).

Table 2.3: Prevalence of mycotoxins in African medicinal plants

Location	AFBs	FBs	OT	P A	Reference
South Africa	x	+	x	x	Sewram et al., 2006.
Egypt	-	-	-	-	Donia, 2008.
South Africa	-	+	x	x	Katerere et al., 2008.
Nigeria	+	-	+	+	Oyero and Oyefolu, 2009.
Nigeria	+	x	x	x	Ezekwesili-Ofili et al., 2014.
Kenya	+	-	-	-	Mukundi, 2015.
Egypt	+	x	x	x	Migahed et al., 2017.
Kenya	+	+	x	x	Keter, 2017.
Kenya	+	+	x	x	Korir et al., 2017.

PA = Penillic acid; Detected (+); Not detected (-); Not tested(x)

Studies on African medicinal plants indicate the limited range in the number of mycotoxins screened, with most studies focusing mainly on aflatoxins and fumonisins. Considering the ecological conditions in Africa and the risk of mycotoxin contamination, there are comparatively few published studies on mycotoxin occurrence in African medicinal plants and their products compared to other regions such as Europe, Asia, and the USA. Various toxigenic moulds produce more than one mycotoxin. As indicated earlier, some mycotoxins are produced by more than one fungal species. Therefore, it is crucial to investigate multiple mycotoxin occurrences in salient medicinal plants as a proactive approach to preventing mycotoxicosis and its associated effects on consumers.

2.10.1 Impact of mycotoxins on public health

Mycotoxicosis refers to the diseases caused by the toxic effects of mycotoxins on animal and human health (Liew et al., 2018; WHO, 2018). Human exposure to mycotoxins can result from ingesting toxin-contaminated plant-derived foods, transferring mycotoxins and their metabolites in animal products, or inhaling toxins in the air and dust. The risk of adverse effects after exposure depends on factors such as type of toxin and purity, dosage, and duration of exposure. In addition, age, sex, weight, diet, exposure to infectious agents, therapeutic substances, and the presence

of other mycotoxins (synergistic or additive effects) can also influence the impact of mycotoxins on human health (Zain, 2011; WHO, 2018; Agriopoulou et al., 2020; Janik, 2020). Factors such as vitamin deficiency, low-calorie intake, alcohol abuse or the presence of an infectious disease can further exacerbate the poisoning severity of mycotoxins (Bennet & Klich, 2003; Zain, 2011; Agriopoulou et al., 2020).

The most important mycotoxins of public health importance include aflatoxins, ochratoxins, deoxynivalenol, zearalenone, and fumonisin (Wagacha & Muthomi, 2008). Human mycotoxicosis can be classified as either acute or chronic. Acute toxicity has a rapid onset and an apparent toxic response. In contrast, chronic toxicity is characterised by low dose exposure over a long period leading to cancer and other generally reversible effects (Tola et al., 2016; Alshannaq & Yu, 2017, Liew et al., 2018; WHO, 2018). Mycotoxins are among the most potent mutagenic and carcinogenic substances known. Protracted exposure through diet has been associated with cancer and kidney, liver, and immune-system disease. Moreover, immune-compromised individuals risk developing the disease due to contamination, which may lead to death (Bennet & Klich, 2003; Tola et al., 2016; WHO, 2018).

The presence of mycotoxins in food and feed worsens the health effects of prevalent infectious diseases such as malaria, hepatitis, and HIV resulting in acute and chronic effects. Individuals exposed to mycotoxins and moulds present symptoms affecting several organs, including the respiratory system, the musculoskeletal system, and the central and peripheral nervous systems. In addition, findings recently suggested mycotoxin involvement in the pathogenesis of autism spectrum disorders (Bennett & Klich, 2003; Ratnaseelan et al., 2018; WHO, 2018). Mycotoxins can inflict many illnesses, as shown in **Figure. 2.9**, such as headaches and various gastrointestinal illnesses, including abdominal pain, vomiting, and diarrhoea.

In the eastern and central provinces of Kenya, 125 people died following a major outbreak of aflatoxicosis in 2004 (Lewis et al., 2005). Hepatocellular carcinoma accounts for about 250 000 deaths annually in Sub-Saharan Africa (Zain, 2011). Assessing the human and animal health risk due to mycotoxin exposures is challenging due to the various factors affecting mycotoxins production or presence in foods or feed. To prevent the adverse health effects of mycotoxins exposure from

medicinal plant products and foods, it is crucial to monitor the quality and safety of herbal raw materials and products.

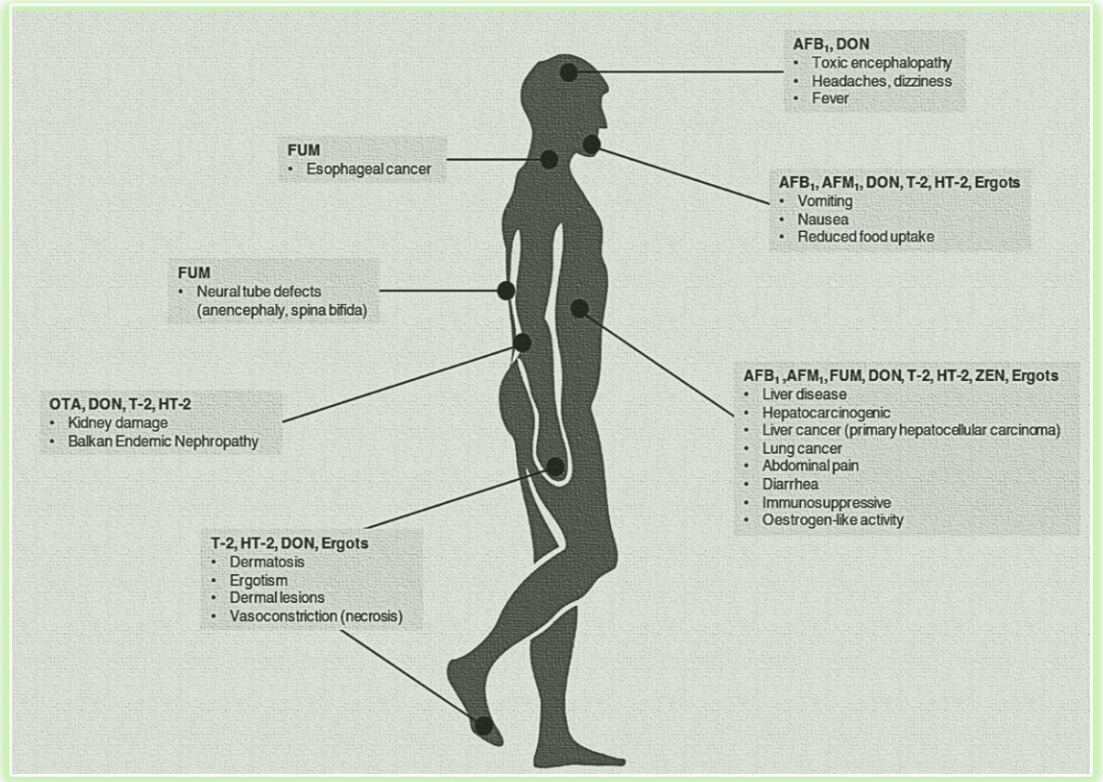


Figure 2.9: Effects of mycotoxins in humans (Cheng, 2022).

2.10.2 Economic implications of mycotoxins

The economic impacts of mycotoxins can be described in different ways taking cognisance of agricultural losses, the effect on trade, human health and mortality, healthcare, and veterinary costs, monitoring, and surveillance, including research and training on mycotoxin prevention control (Zain, 2011). In general, discussions of the economics of mycotoxins are broadly classified in terms of the widespread economic impact of mycotoxins on society and the benefits and costs of mycotoxin control strategies in food (IARC, 2012). Most economic impact studies have been on exposures to one mycotoxin, for example, AFB_1, or FB_1, due to the complexities of global economic impact studies from multiple mycotoxin exposures (Hussein & Brasel, 2001; Zain, 2011).

Mycotoxins lower the value of commodities sold at markets (domestic and export) at the local, regional, national, and global levels. In some instances, these products are condemned due to non-compliance or treatment at an additional cost before being sold for a different use at a lower value (Capcarova et al., 2016; Adeyeye, 2020). The losses incurred mainly affect the producers, suppliers, distributors, governments, and consumers. The losses result in high costs and economic losses for the producing countries. Furthermore, mycotoxin impurities adversely impact veterinary and public health, affecting household security, livelihoods, productivity, and income (Odomkun et al., 2017).

The health economic impacts are mainly attributed to consuming contaminated food, mostly in developing countries during a famine when the households are food insecure and poverty levels are high (Zain, 2011; Perrone et al., 2020). The health care cost is related to the Cost of Illness (COI) concerning the direct healthcare costs, direct non-healthcare costs, and indirect non-healthcare costs of treatment (Jo, 2014; Onwukhugha et al., 2016). Disability Adjusted Life Years (DALY) is another measure of overall disease burden in terms of potential years of life lost due to premature mortality. It includes equivalent years of healthy life lost in states of less than full health, broadly termed disability years (Wu et al., 2010; Lajoie, 2015). Quality-Adjusted Life Years (QALY) assesses public health intervention's monetary value. Worldwide, human health is the most significant impact of mycotoxins, with substantial losses through health care costs and productivity loss and human lives lost (IARC, 2012; McKillop and Sheard, 2018).

Economic losses result from reduced export trade, loss in yield due to toxigenic fungi, reduction in crop value from mycotoxin contamination and low animal productivity from mycotoxin-related health effects (Alshannaq & Yu, 2017; Agriopoulou et al., 2020). When combined, these costs may be staggering (Adeyeye, 2016). Among the impacts of mycotoxins, aflatoxin's economic impact on different crops has been widely studied. In the USA, Vardon et al. (2003) estimated the total annual losses due to aflatoxin, fumonisins, and deoxynivalenol to reach as high as USD 1 billion, while Mitchell et al. (2016) estimated losses ranging from USD 52.1 million to USD 1.68 billion annually in the corn industry. In Senegal, the cumulative healthcare costs of aflatoxins were estimated to be more than USD 92 million of the nation's GDP (ECOWAS, 2014). The economic impact of mycotoxins in medicinal plants is not fully understood, and

literature in that regard is scarce. Whilst studies have focused primarily on food, mainly grains. The role played by other dietary and non-dietary sources, such as medicinal plants, should be considered in future studies.

2.11 Mycotoxin analysis

Analysis of mycotoxins in food and feed involves various steps (**Figure 2.10**): sample preparation, extraction, clean-up, detection, and quantification (Alshannaq & Yu, 2017). Several analytical techniques with different sensitivities and accuracy have been developed for mycotoxin analysis in various matrices—direct methods by chromatography methods such as Thin Layer Chromatography (TLC), Gas Chromatography (GC) or Liquid Chromatography (LC) and indirect methods-(immunochemical) such as Enzyme-Linked Immunosorbent Assay (ELISA).

Indirect methods by ELISA provide a relatively rapid measure of the total mycotoxin content but cannot differentiate between free and masked mycotoxins (Berthiller et al., 2013). The two major approaches to determining masked mycotoxins are chromatographic and immunochemical-based analysis (Anfossi et al., 2016). **Table 2.4** illustrates the studies that have been conducted on mycotoxin analysis of medicinal plants and herbal medicines for the period 2015-2020 with a focus on types of mycotoxins, matrix, extraction solvent/method, clean-up, detection, and quantification methods.

Table 2.4: Common methods used in multiple mycotoxin analysis of medicinal plants and herbal medicines (2015-2020)

Mycotoxin(s)	Matrix	Extraction Solvent/ Method	Clean-up	Detection & Quantification	Reference
OTA, AFB_1, AFB_2, AFG_1, AFG_2	Medicinal seeds	Methanol-Water	SPE column	UPLC-MS/MS.	Chen et al., 2015
Multiclass mycotoxins (AFB_1, AFB_2, T-2)	Ginkgo biloba nutraceutical products	Dilute and Shoot	Sorbents mixture	UHPLC-Obitrap-Z-Sep^+	Martinez-Dominguez et al., 2015
57 Multiple mycotoxins	Plant-based dietary supplements	QuEChERS	___	UHPLC-MS/MS	Veprikova et al., 2015
11 Multiple mycotoxins	Medicinal and edible *Areca catechu*	QuEChERS	___	UFLC-ESI-MS/MS	Liu et al., 2016
Multiple mycotoxins (AFB_1, FB_1, FB_2, ZEN, OTA, T-2, HT-2)	*Alpinia oxypylla*	modified QuEChERS	SPE	UPLC-MS/MS	Zhao et al., 2017
Multiple mycotoxins (AFB_1, DON, FB_1, FB_2, NIV, OTA, ZEN)	Medicinal materials of radix and rhizome	Methanol/water	___	Gold-Immuno-chromatographic assay validated by LC-MS/MS	Hu et al., 2018
Multiple Mycotoxins	*Menthae Haplocalycis*	QuEChERS		UF-LCMS/MS	Luo et al., 2018
AFB_1	Chinese herbs	___	___	Nanoparticle Bio-Bar Code Assay (BCA)	Yu et al., 2018
AFB_1, ZEN	Chinese herbal medicines	Methanol/water	___	Time-resolved fluorescent immunochromatographic assay	Sun et al., 2018

AFs and OTA	Root Herbs from Chinese Markets	Methanol-water	C18 column	UPLC-MS/MS	Su et al., 2018
AFs, ZEN	Edible and medicinal herbs	Methanol-water	IAC	UHPLC-MS/MS	Sun, Yao et al., 2018
Multiple mycotoxins (Aflatoxins Fumonisins, DON, T-2, ZEN)	Functional and medicinal plant species	IAC	QuEChERS	LC-MS/MS	Cho et al., 2019
12 Multiple mycotoxins	Lativia herbal teas	Modified QuEChERS	dSPE	LC-Time of Flight MS	Reinholds et al., 2019
16 Multiple Mycotoxins	Botanicals (Cannabis Sativa L)	QuEChERS	Z-Sep$^+$ dSPE	UPLC-Quadrupole Orbitrap-High-Resolution MS (HRMS)	Narvaez et al., 2020
Multiple (Aflatoxins, ZEN, Ennitians, Beauvericin)	Medicinal plants	Dispersive LLE and QuEChERS	Microextraction	LC-Ion Tap Tandem MS(LC-MS/MS-IT)	Pallares et al., 2020

2.11.1 Methods

Methods regularly used for the analysis of mycotoxins in medicinal plants are thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC) with UV or fluorescence detection (FD), and enzyme immunoassays (EIAs). Recently the qualitative and quantitative determination of mycotoxins has utilised liquid chromatography-mass spectrometry (LC-MS) and gas chromatography-mass spectrometry (GC-MS) (Zhang et al., 2018). Chromatographic techniques coupled to UV and FLD detection are primarily used as confirmatory tests - to test the presence or absence of mycotoxin and to validate results from screening tests for non-compliance with the legislation. Also, chromatography-based methods are used as reference methods for validating Immunochemical based tests. Immunoassays are low-cost and straightforward, yet they are sensitive and selective (Anfossi et al., 2016). Commercially available ELISA-based kits are available for all regulated mycotoxins and provide the most-used analytical tool for assuring food safety throughout the food chain (Pereira et al., 2019).

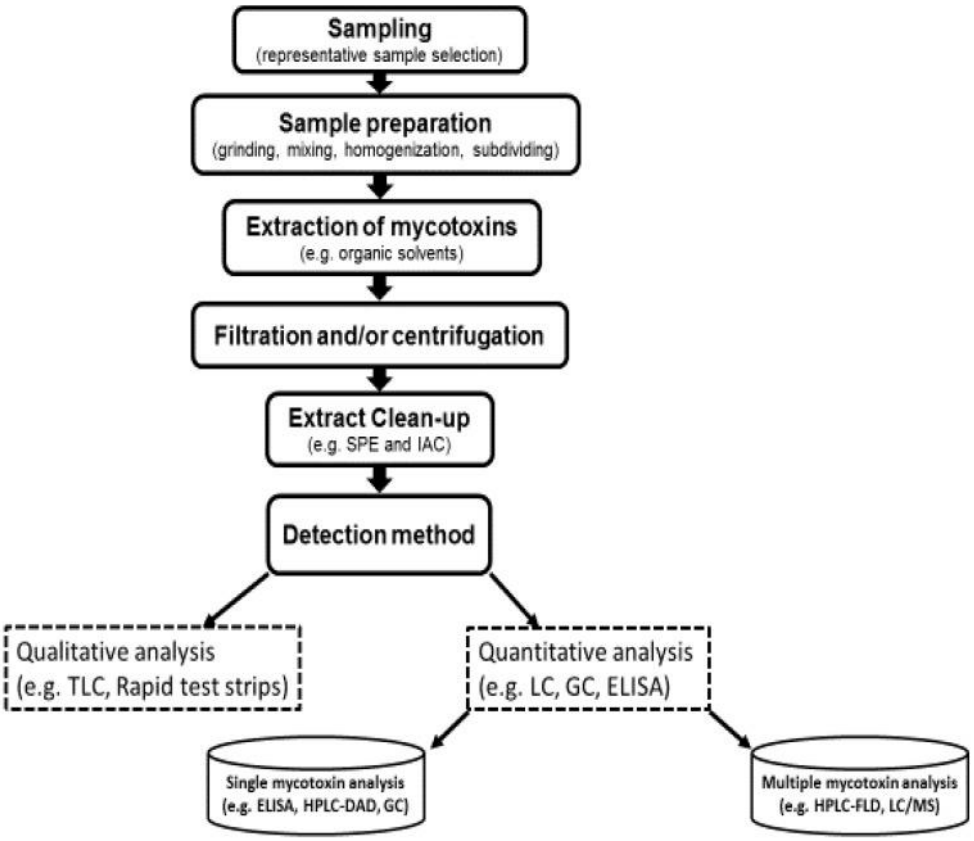


Figure 2.10: Common sequence of mycotoxin analytical steps (Alshannaq & Yu, 2017).

2.11.2 Sampling

Sampling is the most crucial step in mycotoxin analysis. The sampling plan defines the acceptance or rejection limit and mycotoxin test procedure (sampling, sample preparation and analysis). The test procedure consists of various steps (**Figure 2.10**). The sampling procedure specifies quality assurance and regulatory criteria and guarantees the integrity of mycotoxin analysis findings to the sampling lot. It also describes when, where and how to collect samples from the bulk lot, including the sample size (Whitaker, 2003). Furthermore, a sampling procedure includes sample preparation steps and storage conditions, usually below -4°C, to inhibit further microbial growth and mycotoxin production. Due to the heterogeneous distribution of mycotoxins in a matrix, proper sample preparation (grinding, mixing, milling, homogenisation and subdividing) is essential to obtain reliable analytical results (Koppen et al., 2010; Zhang & Banerjee, 2020).

2.11.3 Mycotoxin extraction

The mycotoxin extraction method depends on both matrix effects, mainly physicochemical properties, and the toxins to be analysed (Zhang et al., 2018; Agriopoulou et al., 2020). Several methods have been used for mycotoxin extraction, the most common being liquid-liquid extraction (LLE) and Solid Phase Extraction (SPE). LLE involves using the different solubilities of the mycotoxin in aqueous and immiscible organic phases. The non-target substances are removed in one phase, and mycotoxin extraction in the other solvent (Zhang et al., 2018; Agriopoulou et al., 2020). In comparison, SPE uses both solid and liquid phases to separate the analytes and interfering compounds between a mobile and a stationary phase (Zhang & Banerjee, 2020). As illustrated in **Table 2.4,** several studies have used a methanol-water mixture in mycotoxin extraction.

Compared to LLE, SPE systems are fast and use fewer solvents. They are used to pre-concentrate the sample providing better detection results and sample clean-up procedure (Zhang & Banerjee, 2020). The other method gaining popularity is the QuEChERS (Quick, Easy, Cheap, Effective, Rugged, Safe) which involves an extraction/partitioning step using acetonitrile and salts followed by a clean-up step based on a dispersive solid-phase extraction (dSPE). As illustrated in **Table 2.4**, there is an increased use of QuEChERS in mycotoxin analysis studies of medicinal plants and herbal medicine. The QuEChERS method can extract mycotoxins simultaneously, utilise less solvent, is cost-effective and offers lower detection limits (Singh & Mehta, 2020). Recently, modern automated techniques such as microwave-assisted extraction (MAE) and accelerated solvent extraction (ASE) have been developed (Ashannaq & Yu, 2017; Zhang et al., 2018; Moret et al., 2019).

2.11.4 Purification-clean up

Most often, interfering compounds are found in a sample, and specific techniques are used to clean up the primary samples to achieve more reliable and accurate results. Sample clean-up eliminates substances in the test extract, which may interfere with identifying the mycotoxins (Zhang & Banerjee, 2020). Different mycotoxins require

different cleaning or purification procedures due to their other chemical structures and characteristics. Generally, an extra purification procedure is generally necessary for preparing herbal samples (Zhang et al., 2018). Nevertheless, this further clean-up procedure may not be required for simple matrices after extraction (Bugno et al., 2006). The dilute and shoot approaches, Immunoaffinity Column (IAC), and dispersive Solid Phase Extraction (dSPE) have been used mainly in herbal medicines. Other clean-up procedures include column chromatography, ion exchange columns, Strong Ion Exchange (SAX), and Supercritical fluid extraction columns (**Table 2.4**).

2.11.5 Detection and quantification

Quick, easy, durable, and accurate selective mycotoxin analysis methods allow simultaneous determination of mycotoxins in various matrices. Analytical methods for mycotoxin determination include chromatographic methods such as TLC, (HPLC), (GC), (GC-MS), (LC-MS), (and LC-MS/MS). Immunochemical-based methods include ELISA, biosensors, and fluorescence polarisation (Anfossi et al., 2016; Pereira et al., 2019). In line with this study's objectives, the discussion will briefly focus on the recent techniques, namely HPLC, GC-MS, LC-MS, and ELISA. **Table 2.4** illustrates LC-MS/MS-based methods used to detect and quantify mycotoxins in medicinal plants and products.

Due to their widespread use and applications, immunochemical-based methods can detect more than one compound (for example, a mycotoxin and its derivative), leading to a single result. However, they are selective and not suitable for identifying masked mycotoxins. In comparison, chromatographic methods determine each compound (in an ideal case) as a single parameter. They are also eligible for identifying emerging mycotoxins and masked mycotoxins, although they are expensive, requiring specialised personnel and equipment (Berthiller et al., 2013; Anfossi et al., 2016).

Gas Chromatography is limited to volatile and thermostable compounds and unsuitable for commercial purposes. As illustrated in **Table 2.4**, no studies have utilised Gas Chromatography to analyse mycotoxins in medicinal plants and products. High-performance liquid chromatography (HPLC) offers the advantage of a high-

resolution detection limit, with the possibility to be coupled to multiple automated detection systems (Zhang et al., 2018).

LC-MS is the most precise, accurate, and sensitive method compared to other mycotoxin analysis methods and has become the gold standard for comparing and validating other mycotoxin analysis methods. It has comparatively few simple sample treatment steps and allows for a high throughput of samples (Pereira et al., 2019). LCM-MS is suitable for analysing highly variable mycotoxin compounds, offers low detection limits, the ability to generate structural information of the analytes, and the minimal requirement of sample treatment (Anfossi et al., 2016; Zhang et al., 2018). Recently, ultra-high performance liquid chromatography (UPLC-MS/MS) and Ultra-Fast LC-MS/MS was used to analyse mycotoxins in herbal medicines (**Table 2.4**).

Compared to other methods, immunological methods are employed to determine mycotoxins using polyclonal or monoclonal antibodies produced against the toxin. ELISA is an assay format that uses primary antibodies specific to the mycotoxin analyte or a conjugate of an enzyme-coupled mycotoxin. ELISA has been employed for qualitative, semi-qualitative, and quantitative tests for mycotoxin and screening for the absence or presence above a concentration level of mycotoxin in the sample. ELISA is the likely most used antibody-based assay in mycotoxin analysis of food and feed due to its advantages, including high specificity, simplicity, cheap portability and ability to screen many samples (Anfossi et al., 2016). Other widely recognised methods include nanoparticle-based biosensors; fluorescence polarisation immunoassay microfluidics; phage display (Alshannaq & Yu et al., 2017; Singh & Mehta, 2020). Developing on-site mycotoxin screening assays for herbal raw materials medicines is necessary (Zhang et al., 2018).

The recent trend in using high-performance mycotoxin test procedures incorporating LC-MS-based methods for detection and quantification demonstrates the relevance and importance of this method in future mycotoxin studies of medicinal plants. It is crucial to use a mycotoxin analysis method that offers better accuracy and quick results, considering the costs involved to produce quality results that can be shared with other stakeholders and influence decision-making or provide baseline information for the development of mycotoxin guidelines in marketed South African medicinal plants.

2.12 Mycotoxins standards and regulations

Mycotoxin regulations for food and feed have been established in about 15 African countries out of more than 100 countries worldwide to protect consumers against mycotoxins' harmful effects (FAO, 2004; Misihairabgwi et al., 2019). Mycotoxin levels have been established through various pharmacopoeia for mainly aflatoxins in herbal materials and products. The market for medicinal plants in South Africa remains open and unregulated (Cabuco, 2014). The safety and quality problems due to herbal medicinal products are attributed to a largely unregulated expanding market without adequate safety and quality control systems. There are no established legally binding mycotoxin limits in South African medicinal plants and their products and no specific mycotoxin prevention and control measures in place (Street et al., 2008; Chen et al., 2016; Van Wyk & Prinsloo, 2020).

Through pharmacopoeia, including regional and organisational guidelines, official rules concerning the presence of AFs or OTAs in medicinal plants are shared globally (Zhang et al., 2018). As mentioned earlier in the review, South Africa is an observer member of EU Pharmacopeia and also recognises other pharmacopoeias such as United States Pharmacopeia (USP), British Pharmacopoeia (BP), Pharmacopée française (Ph.f.), Pharmacopoeia Internationalis (Ph.I.), Japanese Pharmacopoeia (JP), Food Chemicals Codex (FCC), Deutsches Arzneibuch (DAB), Deutsches Homöopathisches Arzneibuch (HAB) (Medicines and Related Substances Act, Guidelines, Government Gazette,No 24785, 2003;). According to South African recognised pharmacopoeias, this review will only focus on the mycotoxin limits, namely Ph Eur, BP and USP.

EU, BP, and USP legal limits for AFB_1 in herbal medicines range from 2 to 5 µg /kg, while the limit for total aflatoxins ranges from 4 to 20 µg/kg. The European Pharmacopeia (PhEur) and BP have stricter limits for Aflatoxins (AFs) in herbal drugs, with the limits set at 2 µg/ kg for AFB_1 and 4 µg/kg for total AFs (British Pharmacopoeia Commission, 2012; European Pharmacopoeia Commission, 2016). The United States Pharmacopoeia (USP) has a limit of 5 µg/kg for AFB_1 and 20 µg/kg for total AFs. USP AFB_1 and total aflatoxin limits are almost twice the BP and PhEur limits (USP, 2017).

The absence of South African national pharmacopoeia for mycotoxin limits in medicinal plants and products demonstrates the need for regulations specific to South

African medicinal plants and their products without the general adoption of international pharmacopoeia. The pharmacopoeia's limits recognised by South Africa must be incorporated into the existing legislation to become legally binding and guide mycotoxin quality and safety monitoring strategies for marketed medicinal plants and products.

2.13 Prevention and control of fungi and mycotoxin contamination

Mycotoxins are stable against physical and chemical processes and difficult to eliminate during production, processing, and storage (Zhang et al., 2020). Pre- and post-harvest mycotoxin prevention and control strategies aim to eliminate fungal contamination (primary prevention), inhibit mycotoxin production (secondary prevention), and decontamination and detoxification (tertiary prevention). As previously discussed, commercial trade in medicinal plants is dominated by informal markets that do not have the necessary conditions to deter fungi growth. Furthermore, the stakeholders involved in the medicinal plant's supply do not have adequate knowledge about fungi spoilage and mycotoxin contamination.

Mycotoxin prevention and control measures across the food chain ensure that maximum tolerance limits for mycotoxins in commodities are maintained and safe for consumption (Wagacha & Muthomi, 2008). Prevention and control strategies broadly fall into three categories: physical, chemical, and biological, in order of importance (Zain, 2011). Due to the scarcity of published studies and validated methods for preventing and controlling fungi and mycotoxin contamination specific to medicinal plants, the review will only focus on closely applicable methods to medicinal plants.

2.13.1 Physical strategies to prevent mycotoxin contamination.

Physical strategies are based on the control of environmental factors and storage management. Physical methods mainly aim at the quick reduction in moisture (<12%) prior to storage, primarily achieved by drying, including safe harvesting and storage practices suitable for the agroecological region (Luo et al., 2018; WHO, 2018). Implementing Good Manufacturing Practices (GMP) and processing practices is crucial to ensuring the safety and quality of medicinal plants and their products (Zain,

2011; Van Vuuren et al., 2014). Storage facilities and trading facilities across the supply chain must be capable of maintaining low moisture and temperature whilst preventing contamination by dust, insects, and rodent attacks (WHO, 2018; Agriopolou et al., 2020). Moreover, similar to the food industry, good sanitation practices and segregation of plant materials is essential as different plants, due to different physiochemical characteristics, are prone to contamination by various fungal species (WHO, 2003; Agriopoulou et al., 2020;). For example, the seed of *Foeniculum vulgare* was contaminated with *Alternaria, Mucor* and *Aspergillus* species in contrast to the seed of *Berginia ciliate,* which was only contaminated with *Penicillium* (Aiko & Mehta, 2016).

2.13.2 Chemical strategies to prevent mycotoxin contamination.

The appropriate use of antifungal agents, chemical preservatives, fungicides, and pesticides across the supply chain can help abate fungal infection or insect infestation of crops and subsequent mycotoxin contamination. Chemical methods are either used to prevent fungal contamination or reduce mycotoxins. Chemical treatment was reported as the most effective means of removing mycotoxins from contaminated materials (Agriopoulou et al., 2020). The detoxification procedure should ensure that the toxin can be transformed into a non-toxic form without adverse effects on the raw product. Mycotoxins in agricultural products have been treated chemically using oxidising agents, aldehydes, acids, and alkalis gases (ammonia, hydrogen peroxide, formic acid, sulphur dioxide) (Luo et al., 2018). Itraconazole and amphotericin B fungicides have effectively controlled the aflatoxin-producing Aspergillus species (Ni & Street, 2005). Simultaneous application of chemicals and physical conditions such as moisture content, heat, ultraviolet or gamma irradiation, sunlight and pressure at different treatment periods can enhance detoxification. However, fungicides are being discouraged due to economic reasons, environmental protection, and food safety concerns (Wagacha & Muthomi, 2008; Colovic et al., 2019).

2.13.3 Biological strategies to prevent mycotoxin contamination.

Biological control involves inhibiting one organism's growth, infection, or reproduction using another. The control can be achieved by suppressing the development of toxin-producing fungi or the ability to change mycotoxins or reduce mycotoxins to harmful substances by producing intra- or extracellular enzymes (biodegradation and biotransformation) (Haque et al., 2020). However, atoxigenic bio-control fungi's ability to control toxigenic types depends on the differential effect of macro and micro-climatic conditions on the antagonist–pathogen interaction (Agriopoulou et al., 2020).

Significant developments have been made using bacterial, fungal and yeast applications as biological control agents (Zain, 2011). Notable is the antagonistic capacity of *Trichoderma (T. gamsii 6085*) to reduce deoxynivalenol production by *Fusarium culmorum and Fusarium graminearum* by 92% (Matarese et al., 2012). Several yeast species can degrade toxins to less toxic or non-toxic substances. A yeast suspension of *Saccharomyces cerevisiae* was used in the post-harvest treatment of coffee during processing. It significantly reduced the total incidence of *A. niger* and *A. ochraceus*. The yeast *Trichosporan mycotoxinivorans* can detoxify zearalenone and ochratoxins (Mederiors et al., 2012). Furthermore non -toxin-producing strains of *F. verticillioides* and *F. proliferatum* are better bio-control agents for toxigenic strains (Zain, 2011).

2.13.4 Education and training

There is poor awareness about mycotoxins amongst the general population, producers, manufacturers, and public health professionals in Africa. Also, prevention and control measures, disease management and surveillance do not focus on mycotoxins despite the increased recognition of non-communicable and chronic diseases. Community public health promotion activities aimed at mycotoxins awareness can sensitise the population to mycotoxins risks, prevention, and management (Matumba et al., 2016; Adekoya, 2017; Misihairabgwi et al., 2017).

Public health education and training act as a software component for mycotoxin prevention and control strategies by building capacity for the various stakeholders involved in the medicinal plant supply chain. This will help them recognise risk factors

for contamination with fungi and mycotoxin and take necessary measures to prevent and identify spoiled/contaminated products. The achievement of mycotoxin reduction and control is dependent on concerted efforts by all stakeholders along the supply chain (Sofowora et al., 2013; Faour-Klingbeil & Todd, 2020). The World Health Organisation has developed plans and strategies focusing on field projects, strengthening surveillance and mycotoxin awareness and education in Africa (WHO, 2018). Therefore, there is a need for effective monitoring and surveillance with efficient sampling and analytical methods to reduce mycotoxin risk in Africa (Wagacha & Muthomi, 2008; Zain, 2011).

Other emerging innovative technologies that have been getting worldwide recognition have great potential to be used in fungi and mycotoxin decontamination and mycotoxin detoxification, adsorption, and inhibition of mycotoxin production and elimination. Antifungal nanomaterials/particles (NPs) which have exhibited biocidal effects on fungi include Copper, Silver, and Zinc oxide, among others. For example, Silver NPs inhibited *Aspergillus* growth and aflatoxin production (Zhang et al., 2020). Organic and inorganic mycotoxin binders have been used, such as activated charcoal, oat fibres and alfalfa fibres, silicates, cholestyramine and polyvinylpyrrolidone (Whitlow, 2006).

Combined approaches are essential due to the limitations and applicability of various mycotoxin prevention or control methods and their effects on medicinal plants' phytochemicals and potency. International guidelines to control mycotoxins in food and feed, including medicinal plants and products, have been developed, but practical implementation is lagging (Zain, 2011). There is a need for an integrated or multidisciplinary approach to mycotoxin prevention and control in medicinal plants and herbal medicines. Furthermore, it is paramount to critically evaluate the mycotoxin prevention and control strategies, taking cognisance of the environment, sustainability, cultural acceptability, economic feasibility, ethical implications, and the overall efficacy of potential interventions specific to medicinal plants and their products.

2.14 Conclusion

The literature review has shown a growth in the demand and trade for medicinal plants in South Africa. The market trade of medicinal plants is widespread across South Africa

and is a source of employment for urban and rural populations. The commercial trade in medicinal plants involves various players, from collection to retail. Along the medicinal plant supply chain, various pre-and post-conditions and practices can lead to contamination of medicinal plants with moulds and mycotoxins. Moulds and their mycotoxin present public health and economic impacts. Assays for identifying and quantifying fungi and mycotoxins in medicinal plants have advanced over the years. Recently there has been a shift from traditional methods and techniques to modern molecular-based techniques for fungi identification and LC-MS-based methods for mycotoxin detection and quantification.

Understanding the microbial contamination status of medicinal plants is one of the most important steps to ensuring the safety, quality and efficacy of medicinal plants and their products. The quality of herbal raw materials, prevention of their contamination with fungi and mycotoxin occurrence, and control and testing systems are all essential to reducing the exposure to consumers. What is of immediate importance is to ensure the quality and safety of commercially traded medicinal plants to minimise the risk of mycotoxin exposures. Factors fundamental to a country's ability to protect its population from mycotoxins include the political will to address mycotoxins exposure and the capability to test food for contamination, which determines whether requirements can be enforced. The paucity of information on mycotoxin contamination in medicinal plants and mycotoxicosis in South Africa compared to the extent of the medicinal plant trade points out the need for more detailed studies. Most studies have focused on a few mycotoxins, mostly aflatoxins and fumonisins, but future studies should focus on multiple mycotoxin assessments in medicinal plants and herbal preparations.

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CHAPTER 3
THE MEDICINAL PLANT TRADE IN THE FREE STATE, SOUTH AFRICA

Abstract

This chapter focuses on the medicinal plant trade in the Free State Province of South Africa. The study aimed to characterise the medicinal plant trade regarding the frequently traded species, economic value, traders' social profiles, safety and quality aspects. A market survey was conducted using semi-structured interviews across 29 locations with 48 respondents. The trade in medicinal plants was mainly dominated by street vendors (66.6%) and *muthi* shops (33.3%). Basotho people made up 69% of the traders, with Zulu, Ndebele, Swazi, and people from other nationalities (Nigeria and Malawi) also plying the trade. Traders were primarily men (60%), worked full time with no other income sources, and had been in business for 0.5 years up to 26 years. The mean monthly income for men was ZAR 1825 and for women ZAR 760. A total of 165 ethnospecies belonging to 48 families were the most frequently sold medicinal plants. Salient families for the medicinal plant trade in the province were *Asteraceae* with 13 species, followed by *Liliaceae* (5) and Fabaceae (3). The dominant species were *Hypoxis latifolia, Dicoma anomala, Helichrysum odoratissimum, Elephantorrhiza elephantina, Tulbaghia alliacea,* and *Pentanisia prunelloides*. Roots (46%) and bark (23.6%) formed the bulk of the plant parts sold. There were no significant differences in the prices of medicinal plant portions ranging from ZAR 10 to 30. Two markets in Johannesburg (60%) and KwaZulu-Natal (40%) were the major suppliers of medicinal plants for Free State traders. Generally, the trade was conducted in environmental conditions that were conducive for the contamination of medicinal plant materials with microorganisms or reduced quality. Present study findings demonstrated the importance of commercial trade as a livelihood source and the need for regulating trade.

Key Words: medicinal plants, *muthi* shop, street vendors, Free State, market, survey, trade

3.1 Introduction

The dawn of urbanisation and resultant commercialisation of traditional health care systems coupled with cultural, social, and economic factors have led to increased demand for medicinal herbs and their formulations (Van Wyk & Prinsloo, 2018). An estimated 72% of black South Africans, representing over 80% of households in rural and urban areas, use traditional medicines based on the consumption of medicinal plants (Williams et al., 2013). According to Mander and Le Breton (2006), 25% of all prescription drugs contain some ingredients from medicinal plant materials.

In South Africa, a regular consumer of traditional medicines uses approximately 0.75 kg of plant material annually. Between 35 000 - 70 000 tonnes of plant materials with an estimated market value of 75 - 150 million are consumed yearly (Mander & Le Breton, 2006). Approximately 20 000 tonnes of medicinal plants worth ZAR 2.9 billion are traded in South Africa annually, contributed by 27 million consumers (Mander et al., 2007). The medicinal plant trade has developed into rural self-employment in Africa for resource-poor communities (van Andel et al., 2012). Over half a million people are estimated to be directly involved in the medicinal plant trade in South Africa (Afolayan & Adebola, 2004). According to a later study by Mander et al. (2007), at least 133 000 people are employed in the trade, and the majority are rural women. The value of trade, the number of people involved in the trade and the quantities consumed demonstrate the socio-economic importance of medicinal plants for many communities. Furthermore, it reflects the importance of adopting or using medicinal plant-based medicines to treat various illnesses.

The South African trade in medicinal plants is categorised as informal (street vendors) and formal (*muthi* shops). The formal sector consists of traders, herbalists, and traditional healers, selling raw medicinal plant materials and traditional medicines from *muthi* shops. Commercial gatherers and traders selling plants from the pavements and street markets constitute the informal sector (Williams et al., 2000; Mander & Le Breton, 2006; Moeng & Potgieter, 2011; Rasethe et al., 2019;). Permanent street traders and temporary pension day markets selling medicinal plants have been reported in smaller urban markets throughout the country (Botha et al., 2004). Five large permanent urban *muthi* markets have been reported in South Africa, namely in Johannesburg- Faraday and KwaMai-Mai; Pretoria- Marabastad; Durban- Warwick,

Triangle and Ezimbuzini; and Limpopo- GaMaja (Moeng & Potgieter, 2011; Williams et al., 2013; Khumalo, 2018; Van Wyk et al., 2018; Mbendana et al., 2019).

Market surveys have been conducted mainly to assess the conservation status of medicinal plant species locally utilised in traditional medicine in Gauteng (Williams et al., 1998; 2000; 2007), Limpopo (Botha et al., 2004; Moeng & Potgieter, 2011; Rasethe et al., 2019), Kwazulu Natal (Cunningham, 1988; Mander, 1998; Ndawonde et al., 2007), Eastern Cape (Dold & Cocks, 2002; Goo & Wit, 2015), Western Cape (Loundou, 2008; Philander, 2014); Mpumalanga (Botha et al., 2004). Medicinal plant trade in the North West, Northern Cape and Free State provinces has not been adequately studied, and published articles are not readily available.

Market surveys help to understand the importance of medicinal plants for the country's inhabitants, including socio-economic and sustainability factors influencing the trade (Mander, 1998; Williams et al., 2007). However, the medicinal plant trade in the Free State (FS) has not been reported, and no published studies are available. This study explores the medicinal plant trade in the Free State province for the first time. The study aimed to characterise the medicinal plant trade in terms of the frequently traded species, economic value, traders' social profiles, safety, and quality aspects.

3.2 Materials and methods

3.2.1 Ethics

The survey followed ethical principles in research. The study protocol was approved by the University Research and Innovation Committee of the Central University of Technology, Free State. Ethical clearance was acquired from the Health Sciences Research Ethics Committee at the University of the Free State (No **UFS-HSD2019/1226/2605)**. Participation in the study was voluntary, and informed consent was sought before conducting the interviews.

3.2.2 Study area

The market survey was conducted in Mangaung metropolitan municipality and four district municipalities, namely Xhariep, Thabo Mafuntsanyana, Fezile Dabi and

Lejweleputswa in the Free State Province of South Africa. As shown in **Figure. 3.1** the province is in the geographical centre of South Africa.

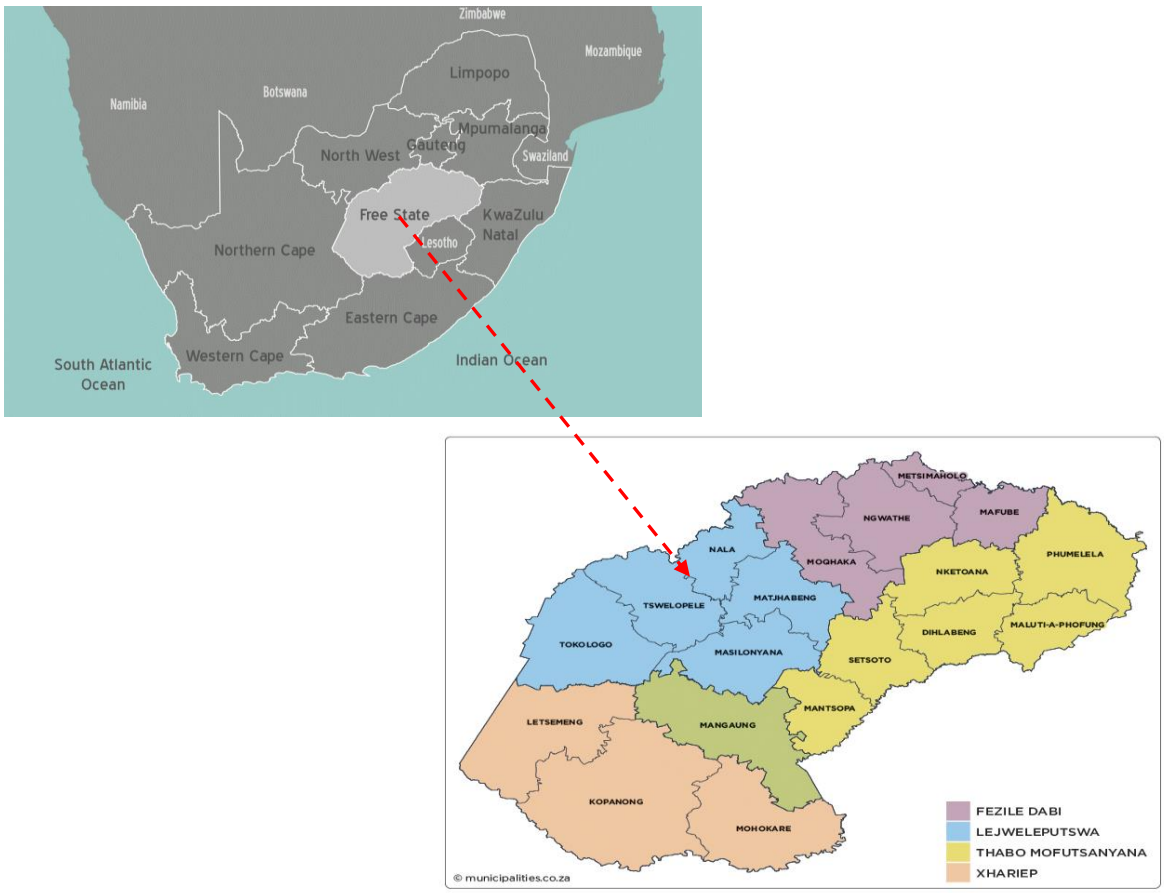

Figure 3.1: Location of the Free State Province in South Africa.
(Municipalities South Africa, 2019)

The Free State is a rural province of farmland, mountains, goldfields, and widely dispersed towns (FS DESTEA, 2014). The climate of the Free State ranges from warm to temperate. Average annual surface temperatures gradually increase from about 14° C in the east to 17° C in the west, with more rainfall in the eastern highlands (1020 mm per year) than in the west (380 mm per year) (Encyclopaedia Britannica, 2020). The grassland biome dominates the Free State province, and its topography consists of escarpments, undulating terrains, plains, and pans. Grassland, Nama Karoo, savannah, and forest biomes consisting of 37 vegetation units make up the province (Rutherford, 2006). The province has a population of 2 834 714 people, according to the community survey report (2016). Mangaung metropolitan municipality has the highest population size compared to other districts, with Xhariep district having the

smallest population. According to Statistics South Africa (2016), the Free State province had an unemployment rate of 38%, which is the highest in South Africa.

3.2.3 Selection of sampling sites and informants

Sampling sites were selected according to municipal regions. One rural and one urban town was targeted per region within each of the five municipalities. The selection of the sites was conducted using purposive sampling to ensure that study sites were widely distributed across the province. Purposive and random sampling were used for nucleated and scattered traders to identify prospective participants (>18 years) and get informed consent before conducting the interviews in each study area. Due to the absence of records for the traders, no appointments were made with them to obtain informed consent (van Andel et al., 2012; Sofidiya et al., 2007).

In areas where the researchers could not locate the first informant, they relied on information from ordinary citizens or other vendors to identify study participants. When researchers randomly asked at least five people and did not mention the availability of vendors or *muthi* shops, the area was classified as not having markets for medicinal plants. The locations surveyed represent 35% of the total towns in the province. The number of interviewees (>30) is satisfactory to generalise study findings to represent medicinal plant trade in the Free State Province as supported by previous studies (Moeng & Potgieter, 2010; Ndob et al., 2016; Mabaleha et al., 2019; Rasethe et al., 2019). Frequently mentioned samples were purchased and analysed for mycological and mycotoxin contamination in **Chapters 4** and **5**.

3.2.4 Data collection and analysis

Data were collected between October and November 2019. Information was compiled through personal observations and semi-structured interviews (Macia et al., 2005). Prior to conducting the interviews, the participants were informed about the significance of the survey and their rights as per the information and consent forms. Semi-structured interviews were conducted using an interview guide to collect data related to the medicinal plant trade in various areas. Informants were interviewed in Sesotho and IsiZulu (the local languages) with the help of interpreters in some cases.

Plant use was not directly relevant to the study; hence no participant was asked about medicinal plant uses (Botha et al., 2004). Personal observations were also made mainly to estimate the number of plant materials in stock, the number of species, storage and hygienic practices, and the trading facilities' condition.

The frequency index, a mathematical expression of the percentage of frequency of mention for a single botanical species by informants, was used for the plants sold in the markets. The following formula was used to calculate the Frequency index (FI).

$$FI = (FC \div N) \times 100$$

Where FC is the number of informants who mentioned the use of the species, and N is the total number of informants (Madikizela et al., 2012). N = 48 in this study. Plant names were documented in the local languages, mostly Sotho and Zulu and scientific names were identified through literature.

3.3 Results and discussion

A total of 48 respondents were interviewed during the survey from 29 locations. Since it was the first kind of survey in the province, some traders were unwilling to participate. Four interviewees were reluctant to give personal information; hence, they were excluded from some parts of the analysis. Therefore, the researchers interviewed as many willing participants as possible in each area.

3.3.1 Market Characteristics

The survey was conducted across 29 locations in the Free State province of South Africa. Twelve places namely Wepener; Reitz Verkeerdevlei, Boshof, Petrusburg, Koffifontein, Jaggersfontein, Kestell, Helibron, Vredefort, Koppies and Frankfort showed no evidence of the trade in medicinal plants during the study period. Areas without shops or street vendors relied on markets in nearby towns or traditional healers to access traditional medicines. Still, the traditional healers only prescribed to their patients and did not sell directly to the public.

Table 3.1: Locations surveyed during the study period

District	Towns Surveyed
Mangaung Metropolitan	Botshabelo, Thaba Nchu, Dewetsdorp, Wepener, Bloemfontein.
Fezile Dabi	Kroonstad, Parys, Frankfort, Sasolburg,Helibron, Vredefort, Koppies.
Xhariep	Jagersfontein, Koffiefontein, Petrusburg, Reddersburg, Zastron.
Thabo Mafutsanyana	Bethlehem, Kestell, Ladybrand, Reitz, Senekal, Clocolan, Phuthaditjhaba,
Lejweleputswa	Brandfort, Winburg, Bultfontein, Boshof, Welkom, Verkeerdevlei,
5 Municipal Districts	**29 locations**

A total of 48 participants were interviewed during the study, including street vendors (32) and *muthi* shops (16). The street vendors mainly worked from the street pavements (sidewalks) in groups at specific vending sites or haphazard around the towns. Their products were displayed in plastic bags or maize sacs (see **Figure 3.2** picture on the right) on the street pavements. Some worked in areas not allocated by the local authority whilst in other areas they worked in local authority council allocated vending places. Menice in Welkom, Bloemfontein-downtown and Ladybrand had the most significant number of aggregated traders i.e., 52% of traders surveyed (n=32). In other areas, the vendors worked in the same street or were scattered at distances of <500 m from each other.

Figure 3.2: Examples of a *muthi* shop trader (left) and a street vendor (right).

Among the *muthi* shops, 8.8% of those surveyed (n=16) were not operating from formal structures but in shacks. *Muthi* shops either sell the unprocessed raw plants or

cut the plant parts into small pieces for easier packaging either in plastics or plastic jars and displayed on labelled shelves or cardboard boxes (see **Figure 3.4**). It was also observed during the study *muthi* shops also sell medicinal plants in combination with other herbal medicines or preparations (see **Figure 3.3).** Most *muthi* shops are not stand-alone businesses, and it is common for *muthi* shops to have a traditional healers' room at the back. Other *muthi* shops only sell medicinal plants, but the traders have a thorough knowledge of medicinal plant uses, preparation and administration; hence they operate more like herbal pharmacies.

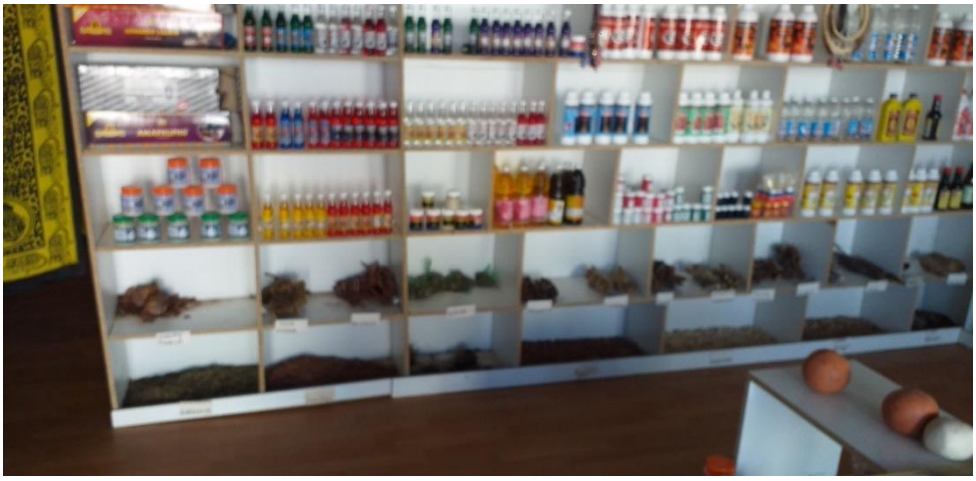

Figure 3.3: Inside a *muthi* shop, on display are raw medicinal plants and herbal products.

This study adds to existing medicinal plant trade knowledge in South Africa by describing the market characteristics within the Free State province, which has not been reported before. Compared to other studies conducted in the Witwatersrand-Gauteng (Williams et al., 2000) and KwaZulu-Natal (Mander, 1998), which reported established permanent markets, this study shows that the trade in medicinal plants was mainly conducted in *muthi* shops and on the street, with no single market. Also, contrary to a study by Williams et al. (2000), who reported the presence of temporary commercial gatherers, the present study findings demonstrate that the traders were mainly full-time permanent traders despite selling on the street. The study findings agreed with Williams et al. (2000), who differentiated traditional medicine trade into formal and informal sectors. The involvement of traditional healers in the medicinal plant trade, reported by Dold and Cocks (2002) in the Eastern Cape province, was not

recorded in the current study as the focus was mainly on the traders who sold directly to the public.

3.3.2 The traders

The traders interviewed during the survey were 60% male and 40% female. The traders' ages ranged from 21 to 68 years, with an average of 39 years. Among the respondents, no male trader was above 60 years compared to women (6,6%). For *muthi* shop traders (n=14), only one owner was below 30 years, with the rest above 30 years to 66 years. The average age for male *muthi* shop traders was 38 years, and for females were 39 years. For street vendors (n=25), age groups ranged from 22 to 68 years. Ten traders preferred not to disclose their ages, hence were excluded from the age analysis. The average age for male vendors was 39 years, and for female vendors, 40. Traders' age group demographic profile has been illustrated (**Figure 3.4).**

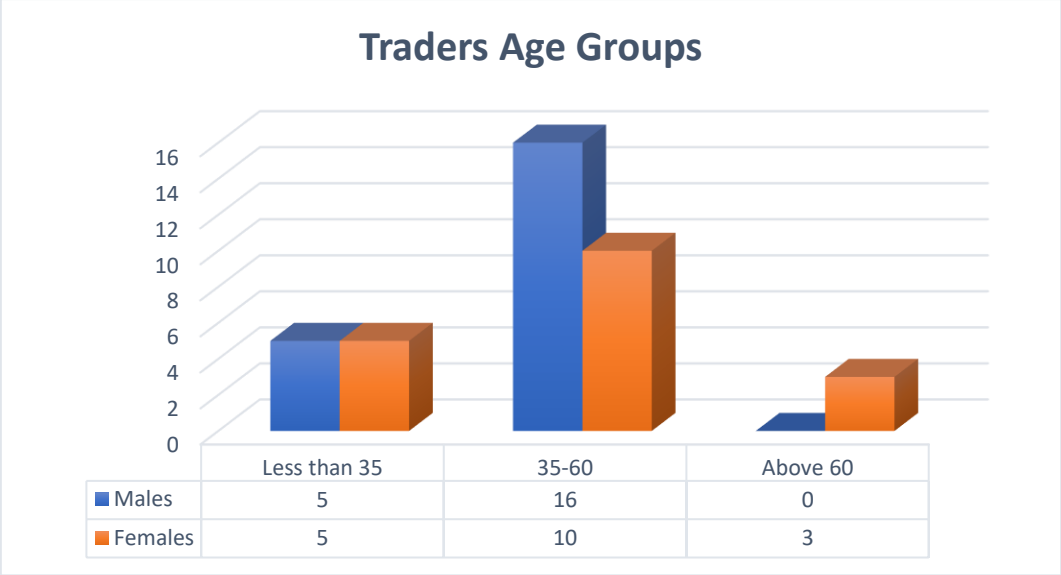

Figure 3.4: Traders' age group demographic profile.

Basotho people dominated the trade, but from the central to eastern parts of the province, there was a mixture of Zulu and Sotho traders. Among the traders (n=48), 69% of the traders were Sotho, Zulu (19%), Ndebele (4%), Swazi (4%), and 4% spoke non-South African languages (Nigeria and Malawi). Most of the traders never attended

school or finished only primary education 75% and 25% went up to secondary level. Of those who went up to the secondary level, 10% were vendors, whilst 15% were *muthi* shop traders.

Contrary to the findings of Mander et al. (1998), Williams et al. (2000), Dold and Cocks (2002), Mander et al. (2007), van Andel et al. (2012), and Towns et al. (2014) who reported the medicinal plant trade to be dominated by women. Study findings indicate that men dominate the medicinal plant trade in accordance with findings reported by Krog et al. (2006), Philander et al. (2014) and Rasethe et al. (2019). Men's dominance of the trade may be attributed to the province's high unemployment rate and illiteracy levels (Sofidiya et al., 2007). Similar to Dold and Cocks (2002) and Sofidiya et al. (2007), medicinal plant traders are mostly illiterate.

The presence of few traders between the age of 18-30 indicate that medicinal plant knowledge is mainly among the older generation and that it takes years of experience to acquire such knowledge. It may also suggest that there is less knowledge transfer to the younger generation, who may prefer modern medicines. The younger generation is also getting better education and has higher chances of obtaining better jobs than the uneducated generation (Oyeyemi et al., 2019). Culturally the trade is primarily dominated by Basotho as they are the largest ethnic group in the region. The study findings highlight the importance of the medicinal plant trade as a source of income in the province for the unemployed and illiterate, consistent with previous studies mentioned above. The involvement of non-nationals from Nigeria, Swaziland, and Malawi also demonstrates the importance of the medicinal plant trade as a livelihood strategy for the indigenous population and other nationalities.

3.3.3 Customers profile

Traders could not distinguish their main customers according to age groups, ethnicity, occupations or type of treatment sought most often. Most of the traders mentioned that both men and women were their customers. Only one *muthi* shop owner said with certainty that they mainly sell specifically to traditional healers. This shop operates more like a wholesale/supermarket where only customers with knowledge of medicinal plants buy at this store.

The current study findings are in accordance with Moeng (2010), who reported the customers to be composed of the general public. In their study, Dold and Cocks (2002) also reported a relatively even number of male and female customers who visited *muthi* shops. More time and resources would be required to describe the medicinal plant customer profiles adequately. Other studies have managed to profile medicinal plant customers; Mati and Boer (2011) reported men; an earlier study by Sofidiya et al. (2007) reported traditional elders and their patients. Since the study was conducted in urban markets, the gender characteristics of the customers might vary due to socioeconomic factors. Generally, study findings indicate that both men and women are the main customers. However, further research might be needed to adequately describe the customer profiles in terms of the plants they prefer, uses, occupations and age groups, including medicinal plant knowledge, which will give an adequate depiction of medicinal plant use or ethnobotany in the province.

3.3.4 Traded species.

Figure 3.5: Stock inside a wholesale *muthi* shop in Thaba Nchu.

Participants were asked to list their top ten selling plants, and a total of 165 ethno-species belonging to 48 families were recorded as the most frequently sold in *muthi* shops and by vendors. The most traded plants in the family are *Asteraceae,* with 13 species, followed by *Liliaceae (5)* and *Fabaceae* (3). The top ten most commonly traded medicinal plants are *Hypoxis latifolia,* followed by *Dicoma anomala,*

Helichrysum odoratissimum, Elephantorrhiza elephantina, Tulbaghia alliacea, Pentanisia prunelloides, Pappea capensis, Alepidea amatymbica, Bulbine narcissifolia, Gunnera perpensa as shown in **Table 3.1**. The table shows only plants with a frequency index of 10 and above, whilst the rest of the data is presented in the **appendices** section.

The external appearance and features of the plant parts sold under the same vernacular names in different *muthi* shops and street vendors were consistent throughout the survey. The number of plants sold by each trader ranged from 20 to 300, with an average of 50. Due to their educational levels, traders in the current study could not ascertain the amounts they sell daily, weekly, or monthly to quantify the plant sold and the income earned. The researchers asked the traders to estimate their estimated monthly income from the trade, which was taken as the economic value of the trade. The traded volumes per species per trader could not be ascertained as it would have involved weighing all pant species and business operations, which could have disturbed business operations and taken more time.

Plants were identified using vernacular names, and the characteristics of plant parts sold between traders and location were consistent, similar to the study by Williams et al. (2000). This may be attributed to the existence of only two main ethnic groups, the Sotho and Zulus considering that the Ndebele, Swazi and other nationalities were also using these local names. However, Dold and Cocks (2002) found using vernacular names unreliable, as considerable differences between place to place and traders were observed.

Table 3.2: Medicinal Plants frequently traded in the Free State province markets.

Scientific name	Family	Local name	FI (%)	Plant part
Hypoxis hemerocallidea Fisch., C.A.Mey. & Avé-Lall.	*Hypoxidaceae*	Ilabatheka	38	Corm root, bulbs
Dicoma anomala Sond.	*Asteraceae*	Hloenya	33	Roots
Helichrysum odoratissimum L. Sweet.	*Asteraceae*	Mpepa/Pefo	31	Leaves, roots and stem
Elephantorrhiza elephantina Burch. Skeels	*Fabaceae*	Mosetsane	31	Roots
Tulbaghia alliacea L.f.	*Alliaceae*	Umwelela/moelela	25	Bulb
Pentanisia prunelloides Klotzsch ex Eckl. & Zeyh	*Rubiaceae*	Setima-mollo	23	Leaves and root
Pappea capensis Eckl. & Zeyh	*Sapindaceae*	Voma	11	Leaves and bark
Alepidea amatymbica Eckl. & Zeyh	*Apiaceae*	Lesoko	19	Rhizome and roots
Bulbine narcissifolia Salm-Dyck	*Asphodelaceae*	Kgomo-ea-badisa	19	Roots
Gunnera perpensa L.	*Gunneraceae*	Qobo	19	Root
Xysmalobium undulatum L.	*Apocynaceae*	Poho-tshehla	19	Whole plant
Eucomis autumnalis Mill. Chitt.	*Asparagaceae*	Umathunga	17	Bulbs and leaves
Clivia miniata (Lindl.) Regel	*Anacardiaceae*	Umayime	17	Whole plant
Eriocephalus Africanus L.	*Asteraceae*	Rosemary/Rosilina	15	Leaves
Drimia depressa Baker Jessop	*Hyacinthaceae*	Moretele	15	Bulb
Aloe ferox Mill.	*Asphodelaceae*	Khala	15	Leaves
Dianthus basuticus Burtt Davy	*Caryophyllaceae*	Hlokoa-la-tsela	13	Roots
Euclea natalensis A.DC.	*Ebenaceae*	Monna-mots'o	13	Roots, bark

Talinum caffrum Thunb	*Portulacaceae*	Punyuka bamphethe/ khutsana	13	Leaves and root
Crotalaria natalia Meisn	*Fabaceae*	Uvelabahleke	10	Leaves and roots
Podocarpus henkelii Stapf ex Dallim. & Jacks	*Podocarpaceae*	Vhulakhuvhaliwe	10	Leaves
Helichrysum caespititium DC	*Asteraceae*	Phate ea ngaka	10	Whole plant
Malva parviflora L.	*Malvaceae*	Tika motse	10	Whole plant
No identification		Setumo	10	
Adenia gummifera Harv.Harms	*Passifloraceae*	Impinda	10	Stems, roots and leaves
Galium capense Thunb	*Rubiaceae*	Mabona	10	Leaves, roots
Cussonia paniculata Eckl. & Zeyh	*Araliaceae*	Moretseng	10	Roots, leaves

The number of plants recorded to be in the top ten frequently sold medicinal plants is similar to Dold and Cocks (2002). They reported 166 medicinal plant species, which provided 525 tonnes of plant materials at R 27 million annually. Moeng (2010) reported 231 medicinal plants for sale by 16 traders, amounting to 0.96 tonnes. This shows that the number of species cannot be used to determine the number of species traded but gives a picture of common plant species and their demand. Two plants, namely *Helichrysum sp* and *Gunnera perpensa,* were also reported as the most popular plants sold at least once a day by Williams et al. (2000). *Eucomis autumnalis* and *Crotalaria* sp are also part of the most frequently traded species were also reported to be among the top ten plants most stocked by Witwatersrand traders *(*Williams, 1996). *Hypoxis hemerocallidea* was reviewed by Dold and Cocks (2002); Street and Prinsloo (2003), and Van Wyk (2015); as among the top ten commercial medicinal plants, likewise this study found that it is the most sold medicinal plant showing its commercial significance.

Present study findings concur with trends observed in the Lowveld medicinal plants' trade, South Africa, where roots were most commonly traded in the Mpumalanga (59.4%) and Limpopo (60.5%) markets (Botha et al. 2004). Previous studies (William et al., 2000; Moeng and Potgieter, 2011; McMullin et al., 2012) also reported roots and bark as the most used plant parts. Similarly, in KwaZulu Natal, bark represented 30.9% of plant parts of market species, and roots represented 32.4% (Mander, 1998). A study in the Witwatersrand (Williams, 1996) reported that bark and roots are the most sold plant parts.

Previous studies show differences in plant use between the various provinces in South Africa influenced by the availability of species and the cultural and market demand. The use of roots from a sustainability perspective is quite destructive compared to other plant parts such as leaves and bark (Mander, 1998). This study was semi-quantitative, only highlighting the most frequently sold plants to assess these for their microbiological safety without going into further detail compared to other authors (Delbanco et al., 2017; Jusu & Sanchez, 2013; Towns et al., 2014; van Andel et al., 2012) who did quantitative economic market surveys.

3.3.5 Economic importance of trade

Figure 3.6: Sample of Setea tea purchased from a *muthi* shop.

The average period in business ranged from 0.5 years to 26 years. Most of the *muthi* shops are owned mainly by men compared to women. Traders were involved in the medicinal plant trade full-time with no other sources of income. The average monthly income from selling medicinal plants was ZAR 1800 for vendors and ZAR 3000 for *muthi* shops. Most traders earned below ZAR 1500 (50%), ZAR 1500-3000 (36.8%), and above ZAR 3000 (13.2%), with mean monthly income for men ZAR 1825 and women ZAR 760. All the traders struggled to determine the amount of revenue they earned from the trade, either daily, weekly, or monthly. Some respondents were hesitant to reveal their actual earnings despite the researchers explaining the purpose of the study; as a result, some traders might have underreported or exaggerated their earnings. The prices of medicinal plants portion ranged from ZAR 10 to 30 and an average of 20 Rands for both *muthi* shops and street vendors. However, the portion sizes were not standardised as they varied between traders and from place to place. Portioning was done without any scales or any form of measurement. Towns et al. (2014) obtained a similar pattern of results. McMullin et al. (2012) reported significant differences regarding quantities and material pricing between traders by location and gender in Kenya.

The involvement of traders in medicinal plants on a full-time basis and sole reliance on the trade as an income source was reported by Williams (2004) in the Faraday market in Johannesburg. Kepe (2007) reported that trade is becoming a key source of cash income for many female-headed households. In this study, the minimum price for a portion was higher than the ones reported by Rosethe et al. (2019) in Limpopo, ZAR 8.50 but was lower than the one reported by Dold and Cocks (2002) in Eastern Cape, which was ZAR 14.9. Prices are influenced by supply, the cost of living and inflation, among other factors. The traders did not have records to document daily and weekly sales, coupled with a lack of financial literacy as evidenced by their inability to state with absolute certainty their actual revenue from the trade (Botha et al., 2007; Williams et al., 2007; McMullin et al., 2012).

Contrary to the present study findings, Dold and Cocks (2002) reported that 62% of the traders earned less than ZAR 500 monthly in the Eastern Cape. Wiersum et al. (2006) also reported that average annual incomes from the medicinal plant trade ranged from ZAR 2400-7200 per year in the Amathole district, which loosely translates to ZAR 200-600 a month. While the earnings differ, this might be attributed to the different economic statuses of the provinces, time, and inflation rates, meaning that the buying power of the earnings might be the same. Another factor could be the demand for medicinal plants in the province; as stated earlier, most traders are full-time with no other sources of income, indicating that they would not be interested in the trade if earnings were insufficient to sustain their household needs.

The study also shows that the traders are permanent, in line with the study by Krog et al. (2012). Similar to other studies (Botha et al., 2007; Williams et al., 2007), this study could not obtain data for accurate measures of the quantities of medicinal plant materials sold or bought as stock material from markets to establish the price per kilogram or economic value of the trade as done by Mander (1998).

The collection of data on retail price, number of plants stocked, and monthly sales, as done in this study, allows for quantification of trade and can assist in approximating and valuing a region's medicinal plant trade (Olsen, 2005; Bussmann et al., 2007). However, it is hard to assess how many medicinal plants are commercially traded, either on a provincial, national, or internationally (Botha et al., 2004). The study has also provided insight into the importance of the medicinal plant trade as a livelihood

source through *muthi* shops and vendors, underlining the importance of the medicinal plant trade for many households since they do not have alternative income sources.

3.3.6 Plant collection and Supply chain

Two markets in Johannesburg (60%) and KwaZulu-Natal (40%) were reported to be the major suppliers of medicinal plants for Free State traders. Nongoma market was also mentioned by the traders who collected or purchased from KwaZulu-Natal and was also reported by Ndawonde et al. (2007). Self-collection was done by 64,4 % of the traders, while others purchased from local collectors. For self-collectors, the distribution was as follows Lesotho (9), Free state (16), and KZN (7). Collection and restocking ranged from 0.1 to 4 times a month with a mean of 2 times a month.

Men (60%) were significantly involved in self-collection compared to women (30%). The local collection was mainly within two to seven-hour walking distances, whilst others were collected from faraway places such as KZN and Lesotho. It was noted that traders who collected or purchased also collected some of their plants locally close to their respective cities, especially Basotho traders. It was also pointed out that even those who collected were also involved in purchasing some plants from other markets. Only one person among those interviewed indicated that they had a permit to collect in the Free State province, and all the plants they sold were collected within the different areas in the Free State. One feature that was noted in Welkom is suppliers (6 females) from KZN who came with dried plant material and supplied the vendors from Durban.

The Free State medicinal plant supply chain comprises traders utilising various sources for acquiring medicinal plants. Traders of Zulu origin mainly get their plants from KwaZulu Natal and Johannesburg markets, whilst Sotho traders primarily collect within the province of Lesotho and partly from Johannesburg markets. Similarly, Botha et al. (2004) reported that all vendors and traders purchased their stocks from gatherers, distant markets, and self-collection in the Eastern Cape. The major suppliers of medicinal plants used by traders have been reported in previous studies, namely Johannesburg (Williams et al., 2000; 2005) and KwaZulu-Natal (Cunningham, 1992; Mander, 1998).

Most medicinal plants sold in the Free State are mostly purchased from markets or collected outside the province, indicating the province's limited biodiversity in terms of species availability. Another factor might be the issue of accessibility and permit systems. Lesotho collectors return home when they renew their temporary visa every month, which might also be a driving factor for out-of-province collection. There is a need for detailed studies of plant species locally available and sourced outside as well as driving factors for purchasing outside to determine the conservation status or propagation measures aimed at supplying the traders with needed plant materials.

3.3.7 Microbiological quality and safety

Safety and quality were assessed qualitatively to identify factors that can cause/lead to fungal and mycotoxin contamination, as discussed in **Chapter 2**. Visual quality checks for the physical presence of contaminates such as dust, poor storage, hygiene and cleanliness of environment were done, and the presence of moulds was checked concurrently with the interviews.

Most respondents reported that they did not know how long it takes for the medicinal plants to expire or lose their efficacy, while some believed that the medicinal plants do not expire. Others mentioned that they would throw them away when they saw that the plants had stayed for too long, more than three months. Whilst some traders insisted that as the plants are dry, they will not get worse, the more they get dry, the more they stay preserved for a long time. Natural drying was the most common preservation method, but the traders brought the plants in dried form. Fresh plants are typically dried at home or the market since they would be exposed to sunlight daily.

Street vendors sell their medicinal plants mainly in small shopping plastic bags in most places, whilst Phuthaditjhaba traders used maize bag sacs, citing their durability. In *muthi* shops, plants were organised in labelled wooden compartments or cardboard boxes. Visible contamination of medicinal plants with dust and soil was observed for most of the plants on sale, especially among street vendors. Plant roots were not washed but just uprooted with their soil and dried in the same state. Some plant materials that we purchased for subsequent experiments (**Chapters 4** & **5**) appeared dry but still had considerable moisture content.

The extensive use of medicinal plants as alternative and complementary medicine means that the risk of contamination, purchase and usage of contaminated natural products is high and is now a public health concern (WHO, 2002; Kosalec et al., 2009; Ekor, 2013). A case study of KwaMai-Mai and Marabastad *muthi* markets in South Africa found that not all traders were knowledgeable and aware of proper drying methods and the effects of microbial contamination. In addition, improper storage, product exposure to direct open air, and use of cheap and unsuitable containers were noted (Mbendana et al., 2019). This was also supported by Rabiou et al. (2019), they reported that exposures to the open air on market shelves and the use of obsolete and substandard packaging are probably the major factors of contamination by yeasts and fungi.

Street vendors mainly displayed their plants in the open, where they risk being polluted by dust, among other things. Collectors, growers and traders largely overlook the proper storage conditions for plant material (Street et al., 2008). The lack of awareness regarding the expiry period for these plants in this study also demonstrates the same. Environmental conditions exposure of the plant material during trade makes them susceptible to microbiological and physical contamination (Kneife et al., 2002). Most of the respondents were full-time traders, and it might be possible that they do not adequately ensure that favourable drying conditions are maintained to prevent microbiological contamination as they would not have adequate time to dry the materials separately but would dry them at their vending space. Araujo and Bauab (2020) reported that suitable dryers are required with air velocity, temperature, and humidity values to ensure optimum moisture content reduction without compromising the medicinal plants' safety and quality. An earlier study of a Faraday *muthi* market in Johannesburg found bacterial contamination in medicinal plants (van Vuuren et al., 2014). Since Free State traders also buy from markets in the same city, there is a risk of the traders purchasing medicinal plants which are already contaminated. Katerere et al. (2008) conducted a study in Cape town and Tshwane to assess fungal and mycotoxin contamination and highlighted the need for studies to cover other urban areas to ensure that consumers are protected. Inadequate post-harvest storage and processing techniques often lead to high levels of microbial contamination (Sher et al., 2014).

Due to a lack of regulatory controls, little attention is paid to product packaging, quality and consumer protection. Therefore, consumer safety is an issue which needs to be monitored to protect the health and well-being of medicinal plant consumers (Ekor, 2013, van Vuuren et al., 2014). Study findings indicate the need for awareness among medicinal plant traders on the importance of proper harvesting, storage, and preservation, as well as maintenance of hygienic environments and the ability to recognise visible pollution and contamination.

3.4 Conclusion

For the first time, the study provides information on the medicinal plant trade in the Free State province, which has not been reported before. The trade in medicinal plants is dominated by permanent vendors (Sotho and Zulu) who mainly sell on the street pavements and *muthi* shops. The dominance of trade by men indicates the importance of trade as a source of income and alternative employment. Most medicinal plant traders are street vendors who conduct their business at unlicenced places with conditions favourable for fungi and mycotoxin contamination. Considering the literacy levels, most of them have barely finished primary education, so they lack basic awareness of food safety and the effects of selling contaminated products. Since the province is supplied by herbs from Johannesburg and KwaZulu Natal markets, the Free State province directly contributes to the medicinal plant trade in the respective provinces.

With most of the traders selling in undesignated places, this highlights the need to regulate the trade by ensuring that vending stalls are constructed where the traders can conduct their business, as this also influences the quality and safety of medicinal plants sold. In addition, the data on the trader's monthly income obtained is not reliable due to different literacy levels and inherent fear factors. Therefore, there is need for capacity building initiatives focusing on financial literacy. Due to the extensive trade in medicinal plants, studies should also focus on sustainability strategies which aim at the conservation of endemic species and cultivation of those species which are mainly sourced out of the province.

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CHAPTER 4
MYCOTOXIGENIC FUNGAL CONTAMINATION IN FREQUENTLY TRADED MEDICINAL PLANTS IN THE FREE STATE PROVINCE, SOUTH AFRICA

Abstract

Medicinal plants sold in markets are prone to contamination by moulds pre- and post-harvest, and some mould species may be capable of producing mycotoxins. The widespread trade and consumption of medicinal plants without safety and quality monitoring make their contamination with toxigenic moulds and their toxins a public health concern. Mycotoxigenic fungal contaminations associated with medicinal plants frequently sold in the Free State of South Africa were investigated to evaluate their safety and quality. Thirty-four medicinal plant samples were purchased from street vendors and *muthi s*hops in the Free State Province (**Chapter 3**). Fungal contamination was evaluated after plating on Malt Extract Agar and Potato Dextrose Agar. Fungal species were isolated as single colonies morphologically and identified using molecular techniques using internal transcribed spacer (ITS) sequencing with extracted DNA. Screening for the mycotoxin-producing potential of the isolates was done using Ultra-High-Performance Liquid Chromatography-Mass Spectrometry (UHPLC-MS/MS).

Approximately 26 % of the samples had no fungi contamination, whilst 74% were only contaminated by one or more fungal species. The mean fungal load in positive samples purchased from street vendors ranged from 1×10^6 to 8.4×10^7, with a mean of 2.55×10^7 Colony Forming Units (CFU/g). Whereas for *muthi* shops, the study reported a mean fungal load of 2.33×10^7 CFU/g, ranging from 1×10^6 to 8.4×10^7 CFU/g. For all positive samples, the fungal burden was above 1×10^6 CFU/g (above the WHO maximum permissible limits for fungal contamination). A total of 54 fungi isolates were recovered, comprising of 17 species belonging to 7 genera clustered into one taxonomic group (Ascomycota). Species belonging to *Penicillium, Aspergillus, Fusarium,* and *Purpureocillium* were the most dominant in the medicinal plant samples. Predominant species isolated from positive samples were mainly *Aspergillus*

niger, Fusarium verticilliodes, Fusarium oxysporum, and *Purpureocillium lilacinum*. From the 54 samples, 17 isolates were selected based on their morphology. About 59% of the 17 isolates tested for their ability to produce mycotoxins were toxigenic, whilst 41% did not produce any detectable quantities of Aflatoxin B_1, Deoxynivalenol, Fumonisin B_1, Fumonisin B_2, Fumonisin B_3, Nivalenol, Ochratoxin A, Zearalenone mycotoxins under investigation. All fungal isolates did not produce, Nivalenol, Ochratoxin A and Zearalenone in culture. The presence of toxigenic moulds presents a potential risk of mycotoxin contamination since the species can produce other mycotoxins not tested for in the study. Standards for the levels of fungi contamination, packaging, proper trading, and storage facilities need to be set, including routine quality monitoring for the commercial trade in medicinal plants.

Key Words: fungi, toxigenic, medicinal plants, South Africa, *muthi* shops, street vendors, mycotoxins, contamination, moulds, liquid chromatography, mass spectrometry.

4.1 Introduction

Since ancient times, medicinal plants have been used for various purposes, including disease treatment by different cultures worldwide. They have been used for their numerous properties, including antimicrobial, nutritional, antioxidant, and other properties (Street & Prinsloo, 2013; Van Wyk et al., 2018). Furthermore, medicinal plants are used in the manufacture of cosmetics, food supplements, preservatives, spices, and herbs (Sofowora et al., 2013; Van Wyk et al., 2018). Their use is rooted in numerous cultural traditions such as African, Amazonian, Arabic Unani, Ayurveda, Kampo, Oriental, Traditional Chinese, and Tibetan medicine among others (Pan et al., 2014; Yuan et al., 2016).).

Medicinal plants play an essential role in basic healthcare systems for up to 80% of the population in developing and underdeveloped countries (Van Wyk & Prinsloo, 2018). This is due to a variety of factors, including limited accessibility, availability, affordability, and cultural acceptability of modern medicines (Maroyi, 2013; Dassah et al., 2018). Medicinal plants provide health and economic benefits to South African indigenous populations. An estimated 60 to 80% of the citizens depend on traditional

medicines involving medicinal plants use for their primary health care needs (Mander et al., 2007, Street & Prinsloo, 2013, Williams et al., 2013).

The growing South African human population, rural to urban migration, poverty and a high unemployment rate have contributed to the development of lucrative medicinal plant trade across the country (Van Wyk & Prinsloo, 2018). Similar trends are evident in the Free State province (as reported in **Chapter 3**), which has one of the highest unemployment rates in South Africa (Schenck et al., 2016; Statistics South Africa, 2019). The preceding chapter showed that the trade in medicinal plants has become a livelihood source for the unemployed rural and urban populace.

The global market for medicinal plants has been rapidly expanding, capitalising on the growing awareness of herbal and aromatic plants worldwide. The medicinal plant extracts segment generated a revenue of USD 27.1 billion in 2016 and is expected to reach USD 44.6 billion by 2024 (Hexa Research, 2017). This growth may be attributed to the belief that these products do not cause overdose, toxicity and have fewer side effects than synthetic drugs (Rocha-Miranda & Venacio, 2019). However, microbial contamination in medicinal herbs is a concern, especially among immunocompromised individuals (Ekor, 2014).

Microbiological quality is an essential feature of healthy products since most of the medicinal plants sold in markets are harvested from the wild (Molim et al., 2016). In developing countries, traditional methods of collection, processing, storage, and distribution without effective control measures for the raw materials of herbal drugs have been reported to influence fungal contamination (Ekor, 2014; de Sousa Lima et al., 2020). Fungal contamination of medicinal plants is more frequent than bacterial contamination due to the ubiquitous nature of fungal spores in the soil and air (Kneifel et al., 2002; Fröhlich-Nowoisky et al., 2009). Contamination mainly occurs pre-harvest due to inadequate drying or during post-harvest storage conditions with favourable humidity and temperature (Muller & Heindl, 2016).

Fungi contamination degrades the quality of raw materials by adversely affecting the chemical composition and thereby decreasing the therapeutic value, shelf life, and market value of products, whilst some fungi species are pathogenic (Singh et al., 2008; Zain, 2011). Numerous groups of fungi, such as *Aspergillus, Penicillium, Fusarium, Alternaria*, and *Claviceps,* have been reported to contaminate medicinal plants. Most

of these fungal species are toxigenic, while others are non-toxigenic, causing spoilage (Pitt, 2000; Altyn & Twaruzek, 2020). Among mycotoxin producing genera, *Aspergillus, Penicillium,* and *Fusarium* have been reported in South African medicinal plants. (Sewram et al., 2006; Katerere et al., 2008). Consequently, medicinal products can be contaminated with mycotoxins which pose a severe risk to public health.

As reported in the previous chapter, the lack of regulation of trade, safety and quality monitoring coupled with trading environment and conditions favour the contamination of medicinal plant materials by moulds at the market. Customers risk purchasing contaminated products from the market and unknowingly use these materials, unaware of the health risks associated with fungi contamination.

Recently several studies have been done on mycoflora in agricultural products (Beukes et al., 2017; Alberts et al., 2019; Phokane et al., 2019; Ekwomadu et al., 2020), with only a few focusing on herbal medicines. The growing use and trade in medicinal plants with no regulation, safety and quality monitoring, and a lack of traders' and consumers' awareness of medicinal plant contamination makes mycoflora studies more pertinent. Thus, the current study evaluated the predominant fungi, the extent of fungal contamination and the presence of mycotoxin-producing fungi in medicinal plants sold in the Free State province of South Africa.

4.2 Materials and Methods

4.2.1 Ethics

Before conducting the study, ethical clearance was obtained from the University of Free State Environment and Biosafety Ethics Committee (Ethics No: **UFS-ESD2019/01831504**).

4.2.2 Materials and Equipment

Potato Dextrose Agar (PDA); Malt Extract Agar, Agar No 2, Thermocycler, sterile pipette tips. ZR Soil Microbe DNA Kit™ (D6001) consists of ZR Bashing Bead™ Lysis Tubes, Lysis Solution (40 ml), Soil DNA Binding Buffer (100 ml), DNA Pre-Wash Buffer (15 ml), Soil DNA Wash Buffer (50 ml), DNA Elution Buffer (10 ml), Zymo-Spin™ IV

Spin Filters (Orange Tops), Zymo-Spin™ IIC Columns, Collection Tubes. NEB OneTaq 2X Master Mix with Standard Buffer (Catalogue No. M0482S), Forward primer (10 µM), Reverse primer (10 µM), Nuclease-free water (Catalogue No. E476), Nano-Drop Spectrophotometer (Thermo Scientific), Acetonitrile, Methanol (VWR International, Zaventem, Belgium) and methanol (Biosolve, Valkenswaard, Netherlands), and Formic acid (≥98) (Merck, Darmstadt, Germany). The mycotoxin standards comprising Aflatoxin B_1 (AFB_1), Zearalenone (ZEN), Nivalenol (NIV), Deoxynivalenol (DON), Ochratoxin A (OTA) were obtained from Sigma-Aldrich (Bornem, Belgium). Fumonisin (B_1, B_2, B_3) were purchased from Promec Unit (Tynberg, South Africa).

4.2.3 Sample collection and preparation

Between October and November 2019, medicinal plant samples consisting of roots, bark, leaves, stems, and bulbs were purchased using purposive sampling to select the traders and locations of *muthi* shops (16) and street vendors (18). After the trader's selection, random sampling was used to determine the portion to buy from the trader. Samples were collected in a dry state in sterile zip lock plastic bags and transported to the Centre for Applied Food Security and Biotechnology Laboratory, Central University of Technology, Free State South Africa. The samples were further dried to reduce moisture content in a laminar air flow dryer (Lasec) at room temperature. All dried samples were milled using Kinematica Polymix (PX-MFC90D) to less than 0.5 mm particle size. Homogenised samples of 30 grams were divided into two for mycotoxin and microbial analysis. Powdered samples were stored at -4 °C to inhibit mycotoxin production and fungal growth until analysis.

4.2.4 Enumeration and Isolation of Fungi

Fungal analytical procedures were performed under aseptic conditions as previously described by Zheng et al. (2017) and Adekoya et al. (2018). For the mycological study, 10g of ground plant material was weighed and suspended in 90 ml of sterilized 0.1% peptone solution in a test tube, thoroughly mixed and serially diluted to 10^{-5}. Using the spread plate technique, an aliquot of 100 µl was inoculated in duplicates on solidified Malt Extract Agar (MEA) with Chloramphenicol (0.1 g/L). Direct plating was also

performed by placing the powder of unsterilised samples on the same medium without dilution. A plate without medicinal plants was prepared parallel and used as the negative control. The inoculated petri dishes were incubated at 25 °C and examined daily for 3 to 5 days, after which fungal colonies were counted using a colony counter (Acolade colony counter). The mean number of fungal colonies appearing on the two plates was taken as the average number of colonies per plate for each plant sample. The number of fungal colonies per gram of sample was calculated and expressed in colony-forming units per gram using the formula:

$$\frac{\text{CFU}}{\text{g}} = \frac{\text{Number of colonies} \times \text{Reciprocal of dilution factor}}{\text{Plating volume(1ml)}}$$

The mean fungal load was also calculated, and the results were expressed as CFU/g of the sample. The incidence of different fungal spp. was assessed by calculating relative frequency and incidence percentage. The relative frequency is defined here as the percentage of samples within a given species found at least once.

After obtaining microbial counts, between 5 and 10 different colonies were selected from those media where growth was observed, ensuring that at least one representative of each morphology was included for further assays. The single colonies were harvested, purified on Agar bacteriological and sub-cultured on Potato Dextrose Agar with Chloramphenicol and then incubated at 25 °C for 7- 10 days.

4.2.5 Morphological observation

A **B**

Figure 4.1: Morphological identification of fungi cultures showing 3-point inoculations on MEA (A) and PDA (B).

The purified isolates were cultured with one and 3-point inoculations for seven days on both MEA and PDA at 25 °C **(Figure 4.1)**. Isolated strains were grouped according to their morphological differences, and one representative from each of these groups was further analysed molecularly by PCR and sequencing of the ITS region. These pure isolates were further analysed for their ability to produce mycotoxins in culture and transferred to cryovials with glycerol (20%) for long-term storage.

4.2.6 Evaluation for toxigenic potential

Seventeen fungi pure isolates representative of the 54 isolates were evaluated for their ability to produce mycotoxins (AFB_1, ZEN, NIV, DON, OTA and FB_1, FB_2, FB_3). A simple solvent extraction method was used with no clean-up due to the high sensitivity of the equipment. Mycotoxins were extracted as described by Sosa et al. (2002) with modifications. Briefly, mycotoxins were tested after growing the pure mould strains on Malt Extract Agar for 7-15 days at 25 °C. Approximately 5 mg of isolate, including the medium, was gently scrapped from the petri dish and extracted in 20 ml of methanol. After centrifugation, the mixture was evaporated at 40 °C in an oven. The extracts were stored at 4° C in the dark until further analysis at Central Analytical Facilities at Stellenbosch University, South Africa.

The extract/residue was reconstituted with 10 ml of water, methanol, and acetonitrile (2:1:1 v/v) and sonicated for 30 minutes. One millilitre (1 ml) of the solution was mixed with 1 ml (75% water and 25% methanol) in 2 ml Eppendorf tubes to dilute the acetonitrile. The mixture was then centrifuged at 12000 rpm for 5 minutes, and 1 ml was aliquoted into the analysis vials for the qualitative analysis of mycotoxins. A Waters Acquity Ultra Performance Liquid Chromatography (UPLC) apparatus coupled to a Waters Xevo Triple Quadrupole Mass Spectrometer (TQMS) (Waters, Milford, MA, USA) was used for high-resolution UPLC-ESI-MS/MS for the simultaneous detection of 8 mycotoxins (AFB_1, DON, NIV, OTA, FB_1, FB_2, FB_3 and ZEN) production by the fungi isolates.

Liquid chromatography separation of 10 µl sample was performed on a Symmetry Waters column UPLC BEH -C18 (100 mm x 2.1 id; 1.7 µm particle size) attached to a guard column (10 mm × 2.1 mm i.d.) (Waters, Zellik, Belgium). The mass spectrometer

was run in the ESI+ mode using multiple reaction monitoring (MRM). High-purity nitrogen was used as the drying and ionisation gas. The capillary voltage was set at 3.50 kV, and the capillary temperature was 140 °C. Mobile phase A consisted of acidified water with 0.1% formic acid (10/1, v/v) and mobile phase B of acetonitrile acidified with 0.1% formic acid (10/1, v/v). A gradient elution program was applied at a flow rate of 0.35 ml/min starting with 98% (0-0.5 min); 98- 60% (0.5-7 min), 60-30% (7-10 min), 30- 5% (10-11 min), 5-0% (11-12 min), 98% (12-12.1 min). Afterwards, an isocratic period of 98% of solvent A was kept for 12.1-14 min.

4.2.7 Molecular identification of fungi isolates.

4.2.7.1 DNA extraction

DNA extraction was carried out using a ZR Soil Microbe DNA kit (D6001) according to the manufacturer's instructions with modifications (Zymo Research Corp). Fungal mycelia (approximately 65 mg) were scrapped off from pure isolates (4-7 days old) and transferred to a 2 ml Eppendorf tube. A 750 µl of Bashing Bead Buffer and beads from ZR Bashing Bead Lysis Tube were added, capped tightly and vortexed at a maximum speed for 5 minutes. The mixture was centrifuged in a microcentrifuge at 10 000 x *g* for 1 minute. The supernatant (400 µl) was transferred to a Zymo-Spin™ IV Spin Filter (orange top) in a collection tube and centrifuged at 7 000 rpm (~7 000 x g) for 1 minute. 1.200 µl Soil DNA Binding Buffer was added to the filtrate in the collection tube. The contents were thoroughly mixed by carefully pipetting slowly. An 800 µl aliquot of the mixture was transferred to a Zymo-Spin™ IIC Column in a collection tube and centrifuged at 10 000 x g for 1 minute. The filtrate was discarded whilst retaining the column, and the remaining flow through processed by repeating the previous step. Using a new collection tube, 200 µl DNA Pre-Wash Buffer was added to the Zymo-Spin™ IIC Column and centrifuged at 10 000 x g for 1 minute. 500 µl Soil DNA Wash Buffer was added to the Zymo-Spin™ IIC Column and centrifuged at 10 000 x g for 1 minute. The Zymo-Spin™ IIC Column was transferred to a clean 1.5 ml microcentrifuge tube. A 70 µl DNA Elution Buffer was added directly to the column matrix to elute the DNA stored at -20 ºC until further analysis. The fungal genomic DNA (10-30 ng/µl) served as a template for identifying the isolates by PCR sequencing using universal ITS1 and ITS4 primers (White et al., 1990).

4.2.7.2 Quality control-Nano Drop

A NanoDrop 2000 spectrophotometer (Thermo Scientific) was used to analyse the purity and yield of DNA extracts. A calibrated pipette was used to dispense 1 µl of DNA elution buffer (ZR DN3004) onto the lower optical surface to perform a blank measurement. Once the blank measurement was completed, both optical surfaces were cleaned using a dry, lint-free lab wipe. After selecting the appropriate constant for the samples to be measured, 1 µl aliquot of DNA sample was dispensed onto the lower optical pedestal. To estimate the DNA concentration and quality, the absorbance was read at 260 and 280 nm using the nucleic acid quantification protocol with sample type defined for double-strand DNA in the (NanoDrop software). The spectral output was reviewed after a sample measurement to assess the sample concentration and quality.

4.2.7.3 Agarose gel DNA electrophoresis

The quality of fungal genomic DNA samples was further analysed on a 0.8% agarose gel. The 0.8% agarose gel was prepared by dissolving 6.4 g agarose in 80 ml 1 x TAE buffer. The solution was boiled in a microwave to dissolve agar until the liquid was clear. The solution was cooled to approximately 60 °C, and 4 µl Ethidium bromide (0.5%) (Sigma Aldrich) was thoroughly mixed into the solution. The agarose solution was poured into the gel tray, combs (20 wells) inserted, and then set for 30 minutes. The comb was removed and placed in the gel tank filled with 1XTAE buffer. Each well was filled with 7 µl of fungal genomic DNA isolates mixed with 2 µl of Gel loading dye (6X bromphenol blue) and 3 µl of NP water. A 2 µl of DNA ladder (Gene Ruler 1 kb) was also put together with the samples in the middle for comparison purposes. The DNA isolates were separated by electrophoresis by running the gel at 85V for 5 minutes and 110V for 20 minutes. The gel was visualised under UV using the BIO-RAD Gel Doc XR + with Image Lab Software (Gel C 200 well plate protocol) **(Figure 4.4)**. This was followed by PCR and DNA sequencing at Inqaba Biotech laboratories, Pretoria, South Africa.

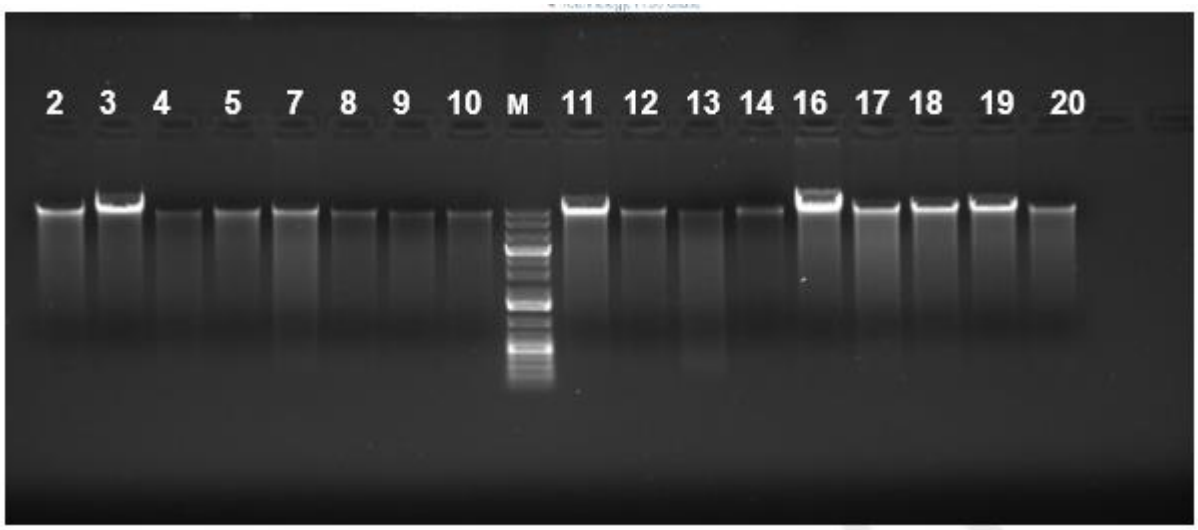

Figure 4.2: Image of agarose gel electrophoresis of fungal Genomic DNA samples: M-DNA Ladder (Gene ruler 1kb)

4.2.7.4 Polymerase Chain Reaction (PCR)

The ITS DNA region of the selected isolates was amplified using universal primers ITS1 and ITS4. The primer sequences used were described by O Donnel and Cigelnik (1997), designed in conserved 5' and 3' regions. The two primers ITS-1 5' -TCC GTA GAA CCT GCG G-3 (forward) and ITS-4 5' TCC TCC GCT TAT TGA TAT GC-3 (reverse) resulted in the amplification of 450 bp elongation factor product. The PCR mix of each sample consisted of NEB OneTaq 2X Master Mix with Standard Buffer (10 µl) (M0482S), Genomic DNA (1 µl), Forward primer (1 µl), Reverse primer (1 µl), 7 µl Nuclease free water (NEB E476). The PCR was performed on Eppendorf 96-well Thermocycler (Eppendorf USA). PCR thermocycler conditions were set as follows: initial denaturation at 94° C for 5 minutes; followed by 35 cycles of -denaturation at 95° C for 30 seconds; annealation at 50 °C for 30 seconds, elongation at 68 °C for 1 min; final elongation at 68 °C for 10 minutes and hold at 4 °C and samples retrieved. The integrity of amplified fragments was analysed by electrophoresis on a 1% agarose gel (CSL-AG500, Cleaver Scientific Ltd) stained with EZ-vision® Bluelight DNA Dye.

4.2.7.5 Purification and Sequencing of PCR Products

PCR products were cleaned using the ExoSAP protocol following the reaction mixture preparation. The ExoSAP master mix was prepared by adding 50 µl Exonuclease I (20

U/µl) (NEB M0293L) and 200 µl Shrimp Alkaline Phosphatase 1 Ul/µl (NEB M0371) to a 0.6 ml micro-centrifuge tube. The reaction mixture, which consisted of 10 µl amplified PCR product and 2.5 µl ExoSAP Mix was mixed well and incubated at 37 °C for 15 min. The reaction was stopped by heating the mixture at 80 °C for 15 min. The extracted fragments were sequenced in the forward and reversed directions (Nimagen, Brilliant Dye™ Terminator Cycle Sequencing Kit V3.1, BRD3-100/1000 USA). The labelled products were cleaned with the ZR-96 DNA Sequencing Clean-up Kit (ZR D4053). The purified fragments were run on the ABI 3500XL Genetic Analyser with a 50 cm array, using POP7 (Applied Biosystems, Thermofisher Scientific). Sequence chromatogram analysis was performed using FinchTV analysis software (Geospiza Inc). Contigs of forwarding and reverse sequence results were assembled using MEGA-X (V 10.1.7) and sequence assembly software and compared with sequences accessible in the GenBank database using the BLAST algorithm (MEGABLAST). Only similarities with a BLAST index of 99% and above were considered for identification.

4.2.8 Data Analysis

Data collected during the mycological and mycotoxin analyses were entered in the Excel function of Microsoft Office to calculate per cent positive samples. MEGA, Chromas, and Finch TV were used to analyse DNA chromatograms.

4.3 Results and discussions

The results of fungal load and the isolated fungal species from each medicinal plant sample from street vendors and *muthi* shops have been illustrated in **Table 4.1.**

4.3.1 Fungal occurrence (fungal load)

Table 4.1. demonstrates the fungal load on medicinal plants sold in the Free State province and species identified. Approximately 26% of the samples had no fungal growth, whilst 24% were only contaminated by one fungal species, and 50% were contaminated by more than one fungal species. The fungal load (CFU/g) in positive samples purchased from street vendors ranged from 1×10^6 to 8.4×10^7 CFU/g, with

a mean of 2.55 × 10^7 CFU/g. Whereas for *muthi* shops, the study reported a mean fungal load of 2.33 ×10^7 CFU/g, ranging from 1 × 10^6 to 8.4 × 10^7 CFU/g. For positive samples, the fungal load was above 1 × 10^6 CFU/g in all samples.

The present study found that positive samples (74%) were contaminated with one or more fungal species. In agreement with the current study findings, Witwoska et al. (2011) also reported fungi contamination in 50% of commercial herb and spice preparations. The study findings also align with Aiko and Mehta (2016), who found 58 out of 63 to contain fungi contamination. Similarly, Chen et al. (2020) evaluated 48 samples and found forty samples (83.3%) containing fungal contamination.

Fungal contamination levels reported in the present study were higher than WHO (2007) maximum permissible limits (1 × 10^3 CFU/g). Esimone et al. (2002) also reported higher fungal loads and found the mean count for positive samples (8) ranged between 5.0 x 10^7 and 4.0 x 10^9 CFU/ml. Other studies have reported lower fungal counts than the present study findings but are still above WHO limits. For example, Kaume (2012) found 24 samples contaminated with fungi at different concentrations, with over one-third of the products not falling within the acceptable range. In another study of medicinal plants from Pakistan, mould contamination was reported in 90% of the samples, with 70% exceeding WHO permissible limits (Ahmad et al., 2014). According to a study by Aiko and Mehta (2016), among the contaminated samples, 47% had a fungal load above WHO limits. Keter et al. (2017) reported that 69% of herbal products did not comply with the acceptable fungal limits as outlined in international Pharmacopoeias.

The fungi load levels observed in the present investigation are higher than those observed by Migahed et al. (2017), who reported fungal counts ranging from 5-100 colonies/g with a maximum of 95-100 colonies. In another study, Omogbai and Ikenebomeh (2013) reported fungal counts in all samples of the herbal teas ranging from 1.1 x 10^2 to 4.5 x 10^5 CFU/g. A most recent study by Ideh and Ogunkunle (2019) found that yeast and mould counts were above 10^4 CFU/g with a mean of 10 $^{5.5}$ CFU/g in traditional oral powdered herbal formulations in Ogbomoso, Nigeria.

Table 4.1: Fungal contamination levels and species in medicinal plants.

Sample Code	Location	Scientific Name	Plant Name (Local)	Total Fungal load per (CFU/ ml)	Isolated Fungal Species
MS02	Parys	*Talinum caffrum* Thunb	Punyuka bamphethe/ khutsana	1.0×10^{6}	*Penicillium biliae*
MS03	Winburg	*Galium capense* Thunb	Mabona	4.7×10^{7}	*Fusarium oxysporum, Aspergillus niger, Penicillium fogi*
MS04	Senekal	*Aloe ferox* Mill.	Khala	3.9×10^{7}	*Aspergillus brasiliensis, Penicillium bransilianium, F oxysporum*
MS05	Sasolburg	*Clivia miniata* (Lindl.) Regel	Umayime	1.0×10^{6}	*Phoma herbarium*
MS06	Sasolburg	*Adenia gummifera* Harv.	Impinda	3.8×10^{7}	*Penicillium corylophilum, Aspergillus niger, Fusarium verticilliodes*
MS07	Qwaqwa	*Eucomis autumnalis* Mill.	Umathunga	0	-NIL
MS08	Ladybrand	*Crotalaria natalia* Meisn	Uvelabahleke	0	-NIL
MS09	Ladybrand	*Scabiosa columbaria* L	Selomi	0	-NIL
MS10	Dewetsdorp	*Tulbaghia alliacea L.f.*	Moelela	2.8×10^{7}	*Aspergillus niger, Purpureocillium lilacinum, Penicillium simplicissimum*
MS11	Dewetsdorp	*Hypoxis hemerocallidea* Fisch. C.A.Mey. & Avé-Lall.	Ilabateka	2.0×10^{6}	*Epicoccum sorghinum*
MS12	Bultfontein	*Hermannia depressa* N.E.Br.	Seletjane	1.7×10^{7}	*Fusarium verticilliodes, Penicillium corylophilum*
MS13	Thaba Nchu	*Bulbine narcissifolia* Salm-Dyck	Kgomo-ea -balisa	6.44×10^{7}	*Fusarium oxysporum, Penicillium fogi, Penicillium biliae*
MS14	Bloemfontein	*Leucosidea sericea* Eckl. & Zeyh.	Cheche (umitshitshi)	1.0×10^{6}	*Fusarium spp*
MS15	Welkom	*Alepidea amatymbica* Eckl. & Zeyh.	Lesoko	0	NIL
MS16	Sasolburg	*Drimia depressa* Baker.	Moretele	3.0×10^{6}	*Penicillium simplicissimum*
SV01	Welkom	*Sclerochiton ilicifolius* A.Meeuse	Molomo monate	5.1×10^{7}	*Aspergillus brasilensis, Fusarium verticilliodes, Penicillium bransilianum*

SV02	Bloemfontein	*Pentanisia prunelloides* Klotzsch ex Eckl. & Zeyh	Sitima-mollo	3.0×10^{6}	*Aspergillus niger*, *Fusarium oxysporum*
SV03	Bloemfontein	*Dicoma anomala* Sond.	Hloenya	1.0×10^{6}	*Fusarium oxysporum*
SV04	Thaba Nchu	*Xysmalobium undulatum* L.	Poho-tshehla/Pohotshehele	4.9×10^{7}	*Fusarium verticilliodes, Purpureocillium lilacinum*
SV05	Bultfontein	*Elephantorrhiza elephantina* Burch. Skeels	Mositsane	1.2×10^{7}	*Penicillium simplicissimum, Phorma herbarium, Aspergillus brasiliensis*
SV06	Zastron	*Helichrysum odoratissimum* L.	Mpepa	0	-NIL
SV07	Zastron	*Dicoma anomala* Sond.	Hloenya	0	-NIL
SV08	Qwaqwa	*Euclea natalensis* A.DC	Monna Motso	0	-NIL
SV09	Bethlem	*Curtisia dentata* Burm.f. C.A.Sm.	Tshwene	1.2×10^{7}	*Penicillium fogi, Fusarium oxysporum*
SV10	Bethlem	*Elephantorrhiza elephantina* Burch. Skeels	Mositsane	0	-NIL
SV11	Senekal	*Cussonia paniculate* Eckl. & Zeyh	Moretseng	1.0×10^{7}	*Penicillium corylophilum, Aspergillus brasiliensis*
SV12	Parys	*Malva parviflora* L.	Phate-ea-ngaka	5.3×10^{7}	*Aspergillus sojae, Fusarium oxysporum, Purpureocillium lilacinum,*
SV13	Kroonstad	*Siphonochilus aethiopicus* Schweinf. B.L. Burtt	Isiphepeto	0	-NIL
SV14	Kroonstad	*Helichrysum odoratissimum* L.Sweet	Mpepa	7.3×10^{7}	*Penicillium corylophilum*, *Penicillium biliae, Epicoccum sorghinum, F verticilliodes*
SV15	Welkom	*Pappea capensis* Eckl. & Zeyh.	Voma	3.0×10^{6}	*Epicoccum sorghinum, A sojae*
SV16	Kroonstad	*Dianthus basuticus* Burtt Davy	Hlokoa-la-tsela	2.0×10^{6}	*Penicillium paneum*
SV17	Kroonstad	*Podocarpus henkelii* Stapf ex Dallim. & Jacks	Vhulakuvhaliwe	3.0×10^{6}	*Penicillium simplicissimum, Penicillium fogi*
SV18	Qwaqwa	*Euclea natalensis* A.DC	Monna-mots'o	1.0×10^{6}	*Penicillium chlamydosporum*
SV19	Bethlem	*Alepidea amatymbica* Eckl. & Zeyh	Lesoko	8.4×10^{7}	*Purpureocillium lilacinum, Aspergillus niger, Fusarium verticilliodes,*

Abbreviations: **SV**-Street Vendor **MS**-*Muthi* shop

The high fungal load can be attributed to the traditional collection, storage, and marketing methods coupled with humid climatic conditions that make medicinal plants susceptible to contamination (Zain, 2011). The high fungal population in the medicinal plants could potentially be due to the environmental conditions observed during the market survey (**Chapter 3**), including unhygienic practices such as displaying plant materials in open and prone to contamination, inadequate drying and poor storage conditions may have promoted the growth of these fungal species. It is a cause of concern that the presence of fungi loads in marketed medicinal plants are above World Health Organisation recommended limits. Considering this, it is necessary for quality control checks of traded medicinal plants and to identify and eliminate such factors that hasten fungal contamination, which is essential to protect consumers from purchasing poor quality products and the effects of fungi contamination on consumer health. Therefore, the present research contributes to a growing body of evidence suggesting that medicinal plants sold by street vendors and *muthi* shops are prone to contamination by fungi before harvesting, after harvesting and during distribution or marketing.

4.3.2 Fungi identification and diversity

The mycological analysis of 34 different medicinal plant samples revealed the presence of various fungal species. The recovered 54 fungi isolates comprised 17 species of 7 genera clustered into one taxonomic group - Ascomycota. The incidence of each isolated genera in positive samples as illustrated in **Figure 4.3**, shows that most of the medicinal samples analysed were contaminated with *Penicillium, Fusarium, Aspergillus, Epicoccum, Purpureocillium,* and *Phorma* species. Predominant species isolated from positive samples were mainly *Fusarium oxysporum* (23.5%) and *Fusarium verticilliodes* (17.6%).

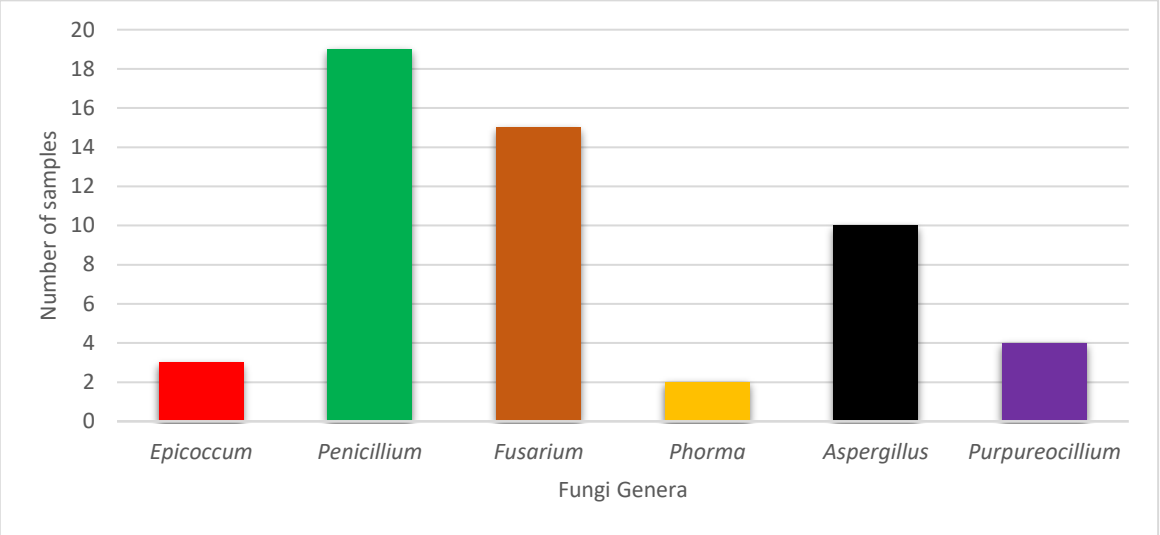

Figure 4.3: Number of contaminated samples per genera.

Table 4.2 presents the identities of isolated species and their toxigenicity. The genetic identity was considered co-specific for most of the isolates when 99% and 100% identities were reached. Regarding species diversity, the genus *Penicillium* was the most predominant with seven isolates, followed by *Aspergillus* and *Fusarium* with three isolates each, whereas others had one species each.

Table 4.2: Isolated fungal species identities and toxigenicity.

SN	Gene Bank Database match	% Identity	Accession No	Toxigenicity
IB02	*Penicillium simplicissimum strain GBS280.39*	99.44	MH856014.1	-
IB03	*Purpureocillium lilacinum isolate END23*	100	KY606536.1	+
IB04	*Phoma herbarum isolate PN2015-06-23A2*	100	KT319052.1	-
IB05	*Epicoccum sorghinum isolate ESCZ020*	100	MN944541.1	+
IB07	*Aspergillus niger strain AUD -C2*	99.82	MN894530.1	-
IB08	*Fusarium sp strain MRC 11718S*	100	MH582471.1	+
IB09	*Epicoccum sorghinum strain LC12103*	100	MN215621.1	-
IB10	*Penicillium fogi strain CBS130047*	99.81	MH865722.1	+
IB11	*Penicillium isolate 148TS (*	100	MN153943.1	+
IB12	*Penicillium corylophilum strain KAS7491 18S*	100	KY469026.1	+

IB13	*Fungal endophyte voucher ARIZ.DM0174*	100	KF673711.1	-
IB14	*Fusarium oxysporum strain Lg3ITS1*	100	KU170716.1	+
IB16	*Penicillium bransilianum strain KAS7779*	99.8	KY469.061.1	-
IB17	*Aspergillus brasielensis*	99.1	MK450632.1	+
1B18	*Penicillium biliae MH1214*	100	LN9011118.1	-
IB19	*Fusarium verticilliodes isolate HYS(1)*	100	MT180471.1	+
1B20	*Aspergillus sojae isolate CBS10092918S*	100	KJ175435.1	+

Data obtained in this study reveal the presence of different fungal species in marketed medicinal plants. While the order of succession of fungal species occurrence (**Figure 4.2**) might differ from earlier studies, the predominant fungi reported in this study, namely *Penicillium, Fusarium* and *Aspergillus,* are consistent with previous studies. The results agree with (Donia, 2008), who found medicinal plants from Egypt contaminated with *Aspergillus, Fusarium* and *Penicillium* more than other fungal genera. Similarly, in their study, Oyero and Oyefolu (2009) also showed that the mycoflora from medicinal plants consisted of six genera: *Aspergillus, Penicillium, Fusarium, Mucor, Alternaria* and *Rhizopus*. Another study also reported the genus *Aspergillus* as the most predominant, followed by *Penicillium, Fusarium, Cladosporium* and *Paecilomyces* (Gupta et al., 2013).

In Nigeria, six fungal genera, namely *Aspergillus, Penicillium, Rhizopus, Cladosporium, Geotricum* and *Candida,* were isolated from 210 medicinal samples (Ezekwesili-Ofili et al., 2014). The present study findings are also consistent with Keter et al. (2017), who found herbal medicinal products sold in Kenya contaminated with the genus *Aspergillus*, followed by *Penicillium*. In another recent study, Omogbai and Ikenebomeh (2013) found all samples of herbal teas contaminated with *Aspergillus niger, Aspergillus flavus, Penicillium expansum, Rhizopus stolonifer* and *Fusarium solanii.* Zheng et al. (2017) also reported a total of 126 fungi strains belonging mainly to the *Aspergillus* and *Penicillium* genera as the predominant contaminants in Chinese medicinal herbs. Whilst there are similarities in the dominant fungal genera reported in other studies, the differences in order of succession might be attributed to factors such as plant age, nutritional and plant water status, and environmental and geographical conditions factors (Zheng et al., 2017). In comparison, fungi from other

genera, including *Phoma* and *Epiccocum* were detected at low incidence in the current study.

Epicoccum sorghinum, a *phoma*-related species, is a major fungal contaminant during pre-harvest and post-harvest stages of various cereals, especially sorghum, and a potent producer of mycotoxins such as tenuazonic acid (TeA) (de Oliveira et al., 2018). The presence of *Epicoccum* species might point to the possibility of cross-contamination from cereals to medicinal plants and the possible contamination of the medicinal plant materials with TeA mycotoxin. This can be attributed to the Free State province being the major producer of sorghum in South Africa (Statista, 2021). Major field fungi genera are *Fusarium, Alternaria,* and *Cladosporium,* whilst storage fungi are predominantly *Aspergillus and Penicillium* (Pitt and Hocking, 2009). *Phoma* is a phytopathogen that rarely causes infections in humans but normally inhabits the marine systems and soil (Bennet et al., 2018). This is the first study in South Africa to report the isolation of *phoma herbarum* in commercially medicinal plants.

Considering that most of the samples tested positive for *Penicillium and Aspergillus,* this could indicate that most commercial medicinal plants are more contaminated by fungi during storage than when they are in the field. Fungal contamination of herbal preparations is mainly caused by poor handler hygiene, inadequate drying, poor storge and transportation conditions (Famewo et al., 2018; WHO, 2018; de Sousa et al., 2020). The presence of multiple fungi contamination and reported toxigenic species in medicinal plants analysed where more than one mycotoxin could be present in the medicinal plants demonstrates the possibility of increased exposure of consumers to multiple mycotoxins. The present study did not determine whether the isolates are pathogens or endophytes or if they are latent pathogens but only evaluated their ability to produce mycotoxins in culture.

4.3.3 Fungi characterisation (screening for mycotoxin producing potential)

Seventeen isolates were tested for their ability to produce mycotoxins in culture. The analysis showed that 59% were toxigenic, whereas 41% did not produce any detectable quantities of mycotoxins under investigation (AFB_1, DON, FB_1, FB_2, FB_3, NIV, OTA, ZEN). All fungal isolates did not produce NIV, OTA and ZEN. AFB_1 was

produced by one isolate of *Aspergillus* and *Fusarium,* and DON was produced by *Epicoccum* (1), *Fusarium* (3), and *Penicillium* (1). FB_1 was produced by *Purpureocillium* (1), *Penicillium* (3), *Aspergillus* (1), *Fusarium* (1); FB_2 by *Purpurecillium* (1), *Penicillium* (3), *Fusarium* (1), FB_3 by *Purpurecillium* (1), *and Penicillium* (2), *Fusarium* (1). The analysis results on the mycotoxin-producing potential of the fungal isolates are presented in **Table 4.3** and **Figure 4.4**. The most predominant toxigenic fungi species belonged to the *Penicillium* (41%), *Fusarium* (20%) and *Aspergillus* (20%).

Table 4.3: Mycotoxin-producing species

Mycotoxin	Toxigenic Species
Aflatoxin B_1	*Aspergillus sojae, Fusarium oxysporum.*
Deoxynivalenol	*Epicoccum sorghinum ESCZ020; Fusarium strain, Fusarium verticilliodes, Fusarium oxysporum.*
Ochratoxin A	NIL
Nivalenol	NIL
Fumonosin B_1	*Aspergillus sojae, Fusarium verticilliodes, Purpureocillium lilacinum; Penicillium fogi, Penicillium isolate, Penicillium corylophilum.*
Fumonosin B_2	*Fusarium verticilliodes, Purpureocillium lilacinum; Penicillium fogi, Penicillium isolate, Penicillium corylophilum*
Fumonosin B_3	*Purpureocillium lilacinum, Fusarium verticilliodes, Penicillium isolate, Penicillium corylophilum*
Zearalenone	NIL

Penicillium, Fusarium and *Aspergillus* species were the predominant toxigenic fungi isolated during the study. These results match those observed in previous studies that reported toxigenic fungal species' occurrence. In a study of Argentinian medicinal herbs, 50% out of 40 isolates were toxigenic (Rizzo et al., 2004). Out of 151 *A. flavus spp.* isolates from various medicinal plant samples, 67 isolates (44.4%) were found to be toxigenic (Migahed et al., 2017). Aikho and Mehta (2016), in their study of medicinal herbs and spices in India, found 28 of 187 fungi isolates toxigenic, which included *Aspergillus flavus* (19) and *Penicillium Citrinin* (9). Chen et al. (2020) found approximately 21.6% of *Aspergillus* and *Penicillium* isolates capable of producing mycotoxins. In an earlier study of South-Eastern Africa plant materials used for ritual

infant protection, Delgado et al. (2011) reported the presence of toxigenic *Aspergillus* and *Penicillium* species.

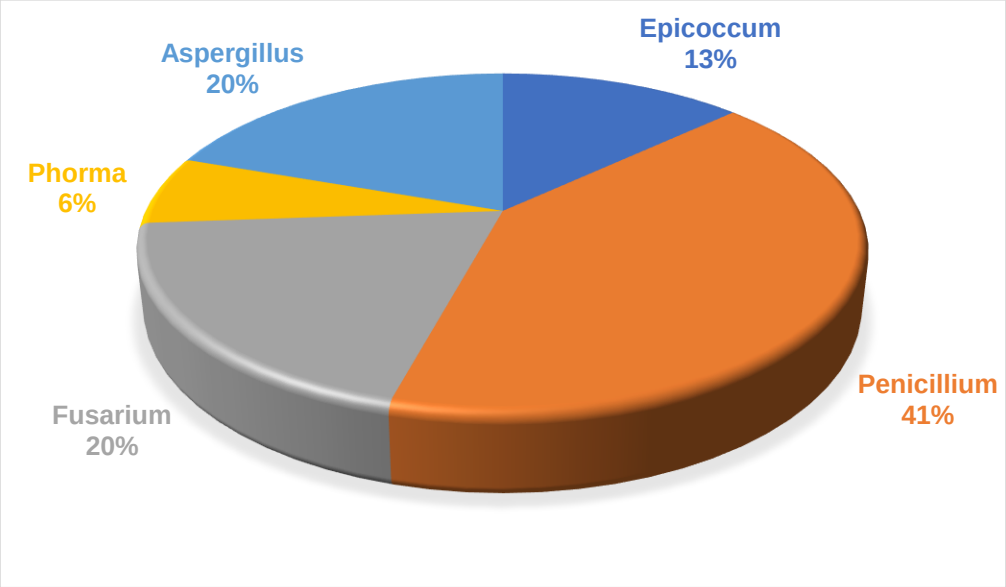

Figure 4.4: Percentage of toxigenic species

Other studies reported the occurrence of other toxigenic species of fungi not reported in the current study. For example, mycotoxin-producing fungi such as *A. flavus, A. ochraceus, F. moniliforme and Penicillium citrinium* were recorded on all herbal samples studied (Kumari et al., 2018). Similar to a study by Su et al. (2018), OTA production was not detected from *A. niger* in culture. In contrast to the present study findings, Katerere et al. (2008) reported traditional medicine samples to be contaminated with three fungal genera: *Aspergillus*, *Fusarium* and *Penicillium*. Still, none contained samples of aflatoxigenic fungi or aflatoxins. Although *Aspergillus, Penicillium,* and *Fusarium* are the primary producers of mycotoxins, not all species are toxigenic (Sweeny et al., 1998).

Whilst *A. niger* did not produce any detectable amounts of mycotoxins; it has been reported to produce fumonisins and ochratoxins (Frisvad et al., 2011; Nielsen et al., 2009). *Penicillium simplicissimum* has been reported to produce the mycotoxins verruculogen, a tremorgenic toxin, although the present study did not evaluate the production of verruculogen (Pitt, 1979). To the best of our knowledge, this is the first

time that *P. simplicissimum* has been isolated from raw medicinal plants in South Africa. *Phoma spp*. produces metabolites, which can be cytotoxic, including cytochalasin A and B, deoxaphomin, proxiphomin and tenuazonic acid (Bennet et al., 2018). *Epicoccum sorghinum* is a facultative plant pathogen and a producer of tenuazonic acid (TA) associated with the haemorrhagic human disorder “onyalay” (Oliveira et al., 2019). In the present study, one of the two isolates of *Epicoccum ESCZ020* tested positive for deoxynivalenol. However, no published studies have reported the production of DON by *Epicoccum* species; hence further studies might be needed to verify this result. The presence of toxigenic *Epicoccum* species in medicinal plants poses a risk to the consumer due to the production of tenuazonic acid and pathogenicity in immune-compromised patients.

Penicillium brasilianum has been reported to be associated with the production of verrucologen and fumitremorgin A & B, penicillic acid viridicatumtoxin (Bazioli et al., 2017). Different strains of *P. brasilianum* have been isolated worldwide from various environmental sources, from soil isolates to endophytes and phytopathogenic (Bazioli et al., 2017). The current study found *P. brasilianum* to be non-toxigenic for the range of mycotoxins tested. Still, its presence may indicate the possibility of cross-contamination of medicinal plants by soil preharvest, during harvesting and presence as an endophyte.

Penicillium bilaiae is a soil borne fungus capable of biodeterioration of organic matter, solubilising mineral phosphates and enhancing plant phosphate uptake (Wakelin et al., 2004). The fungus has been reported to produce mycotoxins, namely ochratoxin A, petrinem A and Patulin. Furthermore, it is used to produce antibiotics such as griseofulvin and penicillin. *Fusarium oxysporum* is commonly found in the soil, and its strains can either be pathogenic or non-pathogenic to plants. It has been reported to produce zearalenone and other trichothecenes (Mirocha et al., 1989). However, strains of *F. oxysporum* isolated in this study did not produce ZEN in culture but however produced deoxynivalenol. *Aspergillus niger* has the potential to produce fumonisins and ochratoxins, which are potentially carcinogenic mycotoxins (Nielsen et al., 2009). In the present study, *A. niger* isolate did not produce any detectable amounts of mycotoxins under analysis.

Aspergillus sojae is a domesticated form of aflatoxigenic species of *A. flavus* and *A parasiticus.* In the present study, *A. sojae* culture tested positive for AFB_1 and FB_1, although other studies have reported that it is non-aflatoxigenic (Frisvad et al., 2019). Differentiating *A. sojae* from *A. flavus* and *A. parasiticus* is inconsistent, and both morphologic and molecular evidence support conspecificity. *A. sojae* strains have been implicated in aflatoxin production. Identifying a strain as *A. sojae* offers no guarantee of its inability to produce aflatoxins or other toxic metabolites (Jorgensen, 2007).

The present study found *Purpureocillium lilacinum* isolate culture positive for fumonisins (FB_1, FB_2 and FB_3). Oliveira et al. (2018) reported the production of aflatoxins and ochratoxin A in untreated surface water by *Purpureocillium lilacinum*, Paecilotoxin isolated from *Purpureocillium lilacinum* is a mycotoxin and a nematocidal metabolite, consisting of serine protease, acetic acid, small chain fatty acids and leucinostatins (Sharma et al., 2016) reported the production of flavoglaucin by *P. lilacinum*.

The inability of some species to produce known mycotoxins may be explained by the laboratory conditions and synthetic medium not optimum for mycotoxin production by the fungi species. Another factor might be that the isolated strains lack the gene required for the biosynthesis of these toxins (Mui-Keng et al., 2003). Therefore, the toxigenic potential must be explicitly determined for individual strains (Jorgensen, 2007).

4.4 Conclusion

The findings of this study indicate that medicinal plants marketed in the study area are contaminated with high fungal levels (above WHO maximum permissible limits) of both toxigenic and atoxigenic fungi species. The presence of toxigenic fungi indicates that medicinal plants may be contaminated with mycotoxins which pose a health risk to the consumers. Furthermore, some of these organisms can cause infections in immunocompromised patients, others are phytopathogens, and some are spoilage fungi which can reduce the therapeutic value of the medicinal plants. The contamination could have resulted from contaminated soil, plants, inadequate drying, unhygienic modes of preparation and prolonged storage. Considering the widespread utilisation of medicinal plants and herbal products as alternative medicines and

associated health risks for consumers. It is necessary to put in place regulatory and legislative frameworks that allow for developing policies and regulations to enable routine safety and quality monitoring and surveillance of medicinal plants and their products sold in formal and informal markets in South Africa. Furthermore, institutions of higher learning should form partnerships with local authorities in conducting tailored food safety and quality awareness trainings among medicinal plant traders.

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CHAPTER 5
IDENTIFICATION AND QUANTIFICATION OF MULTIPLE MYCOTOXINS IN MEDICINAL PLANTS USING ULTRA HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY (UPLC-ESI-MS/MS)[1]

Abstract

Medicinal plants are important in the South African traditional healthcare system; the consumption growth has led to increased trade through *muthi* shops and street vendors. Due to the complex supply chain involved in harvesting, processing, storage, and trade, medicinal plants are prone to contamination with fungi and mycotoxins. Based on mycotoxins' adverse effects, this study investigated multiple mycotoxin contamination using Ultra High-Pressure Liquid Chromatography-Tandem Mass Spectrometry (UPLC-ESI-MS/MS) for the simultaneous detection of AFB_1, NIV, DON, FB_1, FB_2, FB_3, NIV, OTA and ZEN in marketed medicinal plants. A total of 34 samples of commonly sold medicinal plants were purchased and analysed for the presence of mycotoxins. DON and NIV were not detected in the medicinal plant samples analysed. Ten out of 34 samples tested positive for mycotoxins as follows; AFB_1(10.0%); OTA (10.0%), FB_1 (30.0%), FB_2 (50.0%), FB_3 (20.0%) and ZEN (30.0%). Mean concentration levels ranged from AFB_1 (15 µg/kg), OTA (4 µg/kg) FB_1 (7-12 µg/kg), FB_2 (1-18 µg/kg), and FB_3 (1-15 µg/kg), ZEN (7-183 µg/kg).

Multimycotoxin contamination was observed in 30 % of the positive samples with Fumonisin derivatives (FB_1, FB_2, FB_3). The concentration of AFB_1 reported in the current study is above the European Commission permissible limit for AFB_1 of 5µg/kg. Fumonisin concentration did not exceed the limits set for raw maize grain (4000 µg/kg of FB_1 and FB_2). ZEN and OTA are not regulated in South Africa but reported concentrations were below European Commission set limits. Generally, ZEN had the highest mean concentration of 183 µg/kg in medicinal plants suggesting high risk and

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exposure. The findings indicate the prevalence of mycotoxin contamination in frequently traded medicinal plants that poses a health risk to consumers due to mycotoxin's additive or synergistic effects. Therefore, there is a need for routine monitoring of multiple mycotoxin contaminations, human exposure assessments using biomarker analysis, and the establishment of regulations and standards. This is the first report on multi-mycotoxin contamination in Free State marketed medicinal plants using UPLC-ESI-MS/MS.

Key Words: Medicinal plants, contamination, mycotoxins, street vendors, *muthi* shops, UPLC-ESI-MS/MS, Fumonisins, aflatoxins B_1, Zearalenone, Ochratoxin A .

5.1 Introduction

There has been a steady increase in demand for medicinal plant herbs and preparations as complementary and alternative medicine (CAM) and traditional medicine both in developing and developed countries (Ekor, 2013). In developed countries such as the UK, France, Germany and the United States, between 25% and 70% of the population rely on complementary and/or alternative medicine (CAM) (Egan et al., 2011). In Africa, the use of medicinal plants and consumption is significantly higher, around 80%, due to economic, social, and cultural factors (Mahomoodally, 2013; Khan & Ahmad, 2019). Medicinal plants play a vital role in disease prevention, their promotion and use complements current prevention strategies under the Primary Health Care Approach (Sofowora et al., 2013).

The commercialisation of medicinal plants has tremendously increased in many ways because of their widespread use in the manufacture of pharmaceuticals, nutraceuticals, herbal remedies, food supplements, perfumes, and cosmetics (Nasri et al., 2014; Pan et al., 2014). The trade in herbal raw materials and products is growing at an annual growth rate of about 15% (Ahmad & Khan, 2019). Due to a complex supply chain involving different players and conditions from pre-harvesting, harvesting, storage and trade, medicinal plants are prone to infestation by pests, microbes, and toxins (Sitole et al., 2017; Yu et al., 2021). As discussed, and reported in the previous chapter, the environmental conditions and facilities used for the medicinal plants promote the contamination of commercial medicinal plants with fungi, as supported by the fungal load levels reported. Therefore, because of the co-

occurrence of toxigenic fungi species, the contamination of medicinal plants with multiple mycotoxins is highly likely.

Mycotoxins are toxic fungal secondary metabolites and are common contaminants of food, feed. Contaminations are more common in developing countries with poor crop storage and production technologies and climatic conditions which influence fungal growth and toxin production (Agriopoulou et al., 2020). There are over 400 mycotoxins known today. Aflatoxins, ochratoxins, fumonisins, and trichothecenes are the major classes of mycotoxins that have been recognised as of public health significance due to their high occurrence and associated carcinogenic properties (Wu & Gong, 2010; Yu et al., 2021).

Whereas mycotoxin exposures occur via various routes of entry, such as oral, dermal, respiratory, and parenteral, the oral/ingestion route is the major route of entry for mycotoxin exposures. Acute and chronic mycotoxicosis can be developed depending on an individual's susceptibility, the type of mycotoxin and dosage (Zain, 2011; Agriopoulou et al., 2020). Approximately a third of all cases of liver cancer in Africa are due to chronic exposures to mycotoxins (Gibb et al., 2015). Some mycotoxins are carcinogenic mutagenic, teratogenic estrogenic haemorrhagic immunotoxic, and nephrotoxic. hepatotoxic, dermatoxic and neurotoxic (Brase et al., 2009; Bueno et al., 2014).

Previous studies have provided evidence of a positive correlation between fumonisin exposure and high incidences of human oesophageal cancer (Marasas, 2001). Despite the reported and potential impacts of mycotoxins, including their relation to many diseases, they are poorly studied in South African medicinal plants sold in markets where they are prone to contamination (Sewram et al., 2006; Katerere et al., 2008). The control of mycotoxins is inadequately funded, and many African governments do not prioritise mycotoxin control in medicinal plants (Ezekiel et al., 2019).

Most African countries are susceptible to different mycotoxigenic fungi due to a lack of awareness of mycotoxins, poor surveillance and absence of regulations on mycotoxins (Kabede et al., 2020). Few studies have been reported on mycotoxin occurrence in medicinal plants in South Africa (Sewram et al., 2006; Katerere et al., 2008), Kenya (Mukundi, 2015; Keter et al., 2017; Korir et al., 2017); Nigeria (Efuntoye 1999; Oyero

& Oyefolu, 2009; Ezekwesili-Ofili et al., 2014; Ikeagwulonu et al., 2020); Egypt (Aziz et al., 1998; Abou-Arab et al., 1999; Allam et al., 2008; Abol-Ela et al., 2011).

Most of these studies have focused mainly on aflatoxin and fumonisin contamination. Given the increasing demand and trade of medicinal plants and health risks from fungal contamination and their toxins, there is a need to have a broad understanding of the prevalence of mycotoxins in commercially traded medicinal plants. Regrettably, there is limited information on mycotoxins in medicinal plants in South Africa, which is not commensurate with the escalating economic value of the trade. As mentioned earlier, previous studies have been done in South Africa, but no published studies have been done on mycotoxin contamination in medicinal plants sold in the Free State province *muthi* shops and by street vendors; hence there is no information available. Therefore, the present study aimed to assess the safety of medicinal plants concerning multiple mycotoxin contamination (AFB_1, OTA, ZEN, DON, NIV, FB_1, FB_2, FB_3) as recommended by Keter et al. (2017). The study findings will potentially assist in advocating for consumer safety and quality monitoring as well as in developing strategies to maintain the safety of medicinal plants sold in markets.

5.2 Materials and Methods

5.2.1 Standards and reagents

Mycotoxin standards comprising of aflatoxin B_1 (AFB_1), zearalenone (ZEN), nivalenol (NIV), deoxynivalenol (DON), ochratoxin A (OTA) were obtained from Sigma-Aldrich (Bornem, Belgium). Fumonisin (B_1, B_2, B_3) were purchased from Promec Unit (Tynberg, South Africa), acetonitrile (VWR International, Zaventem, Belgium) and methanol (Biosolve, Valkenswaard, Netherlands), formic acid (≥98%) (Merck, Darmstadt, Germany). All reagents were of analytical grade.

5.2.2 Sample collection and preparation

A total of 34 samples were purchased from *muthi* shops and street vendors between October and November 2019. Purposive sampling was used to select the frequently sold medicinal plants whilst random sampling was used to select only a small quantity

from the bulk lot since most medicinal plants were sold without standard portioning or packaging (Romagnoli et al., 2007; Zhang et al., 2018). Sixteen (16) samples were procured from *muthi* shops, whilst 18 were bought from street vendors. They are comprised of roots, barks, leaves, stems, and bulbs. Samples were collected in a dry state in sterile zip lock plastic bags and immediately transported to the CAFSaB laboratory at the Central University of Technology. Samples were further dried to reduce moisture content in a laminar air flow dryer (Lasec). All dried samples were milled using Kinematica Polymix (PX-MFC90D) to less than 0.5 mm particle size. Homogenised samples of 30 grams were divided into two for mycotoxin and microbial analysis. Samples were stored in sterile zip lock bags at -4° C to inhibit mycotoxin production and fungal growth until analysis.

5.2.3 Mycotoxin extraction

A simple solvent extraction method with no sample clean-up was used, as described by Spanjer et al. (2008). Homogenised samples were accurately weighed (approx. 5 g) using an analytical balance (3dp) into a 50 ml tube. Extraction solvent 20 ml of water/methanol/acetonitrile (2/1/1, v/v) was added and sonicated for 60 minutes. 1 ml of sample was aliquoted into a 2 ml Eppendorf tube, double diluted with 75% water; 25% methanol solvent and centrifuged for 5 minutes at 13000 rpm. 1 ml of diluted sample was aliquoted into an analysis vial for analysis.

5.2.4 Equipment Calibration

A calibration graph plotted the obtained peak area or height for each standard working solution against the mass of each mycotoxin injected. Each mycotoxin peak in the chromatogram was identified by comparing the retention times with those of corresponding reference standards. The quantity of mycotoxins in the injected eluate was determined by comparison to the respective standard curves of each mycotoxin standard**. Figure 5.1** illustrates the calibration curves for some of the mycotoxins investigated (FB_2 and ZEN).

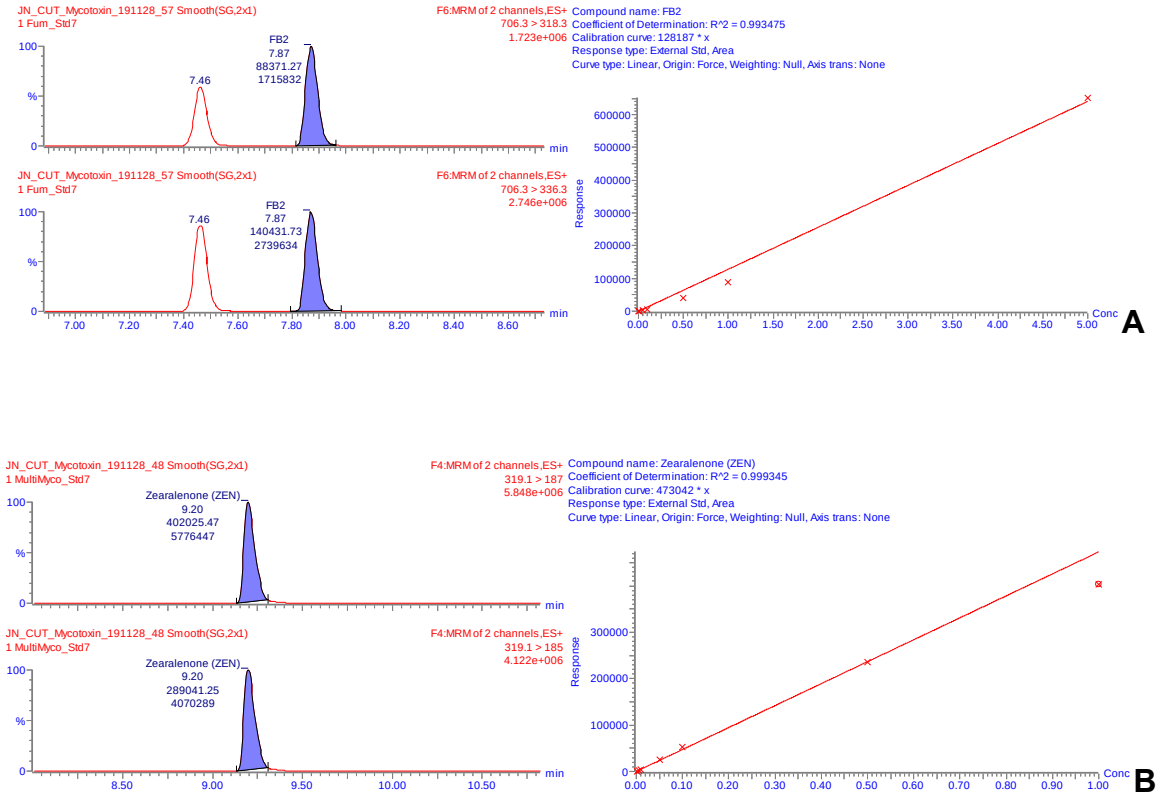

Figure 5.1: Chromatograms and calibration curves for mycotoxin standards A - FB_2 and B-ZEN.

5.2.5 Liquid Chromatography-Tandem Mass Spectrometry

A Waters Acquity Ultra Performance Liquid Chromatography (UPLC) apparatus coupled to a Xevo Triple Quadrupole Tandem Mass Spectrometer (TQMS) (Waters, Milford, MA, USA) was used for high-resolution UPLC/MS/MS for the detection and quantification of mycotoxins. A symmetry Waters column UPLC BEH -C18 (100 mm x 2.1 i.d; 1.7 µm particle size) attached to a guard column (10 mm × 2.1 mm i.d) (Waters, Zellik, Belgium) was used. A fixed sample injection volume of 2 µl was used. Mobile phase solvent A consisted of acidified water with 0.1% formic acid (10/1, v/v) and mobile phase solvent B of 0.1% formic acid in acetonitrile acidified with 0.1% formic acid (10/1, v/v). Multiple mycotoxins were separated in the mass spectrometer operated using selected multiple reaction monitoring channels (MRM) in positive electrospray ionisation mode (ESI+). ESI conditions were optimised: capillary voltage,

3.5V; cone voltage range, 15-50V; collision energy range, 10-40eV; source temperature, 140 °C. Nitrogen was used as the desolvation gas, desolvation temperature - 400 °C; desolvation gas, 800 L/h and cone gas, 50 L/h.

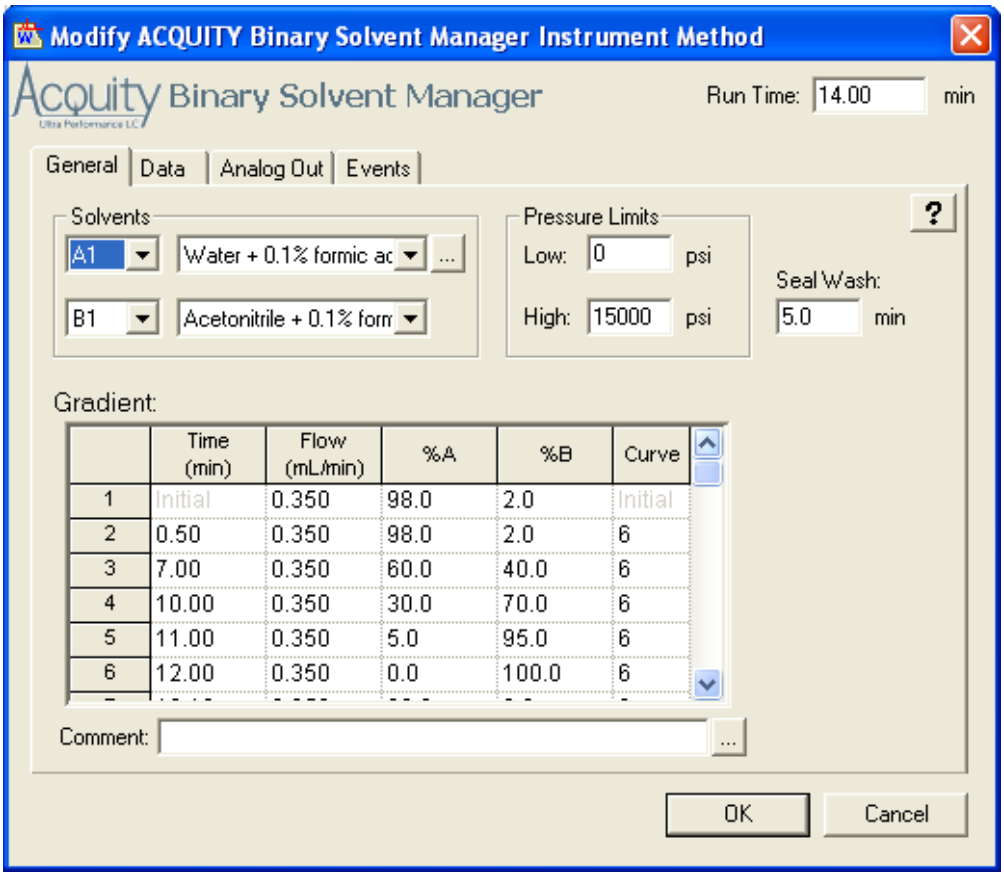

Figure 5.2: Gradient elution program.

A gradient elution program illustrated in **Figure 5.2** was used at initial conditions of 98% A, held for 0-0.5 min at a flow rate of 0.35 ml/min. This was proceeded by a slow gradient change of solvent A to 60% from 0.5-7 min. From 7-10 min, there was another gradient change of solvent A to 30%. A rapid gradient change ensued for solvent A to 5% from 10-11 min. There was another gradient change of solvent A to 0% from 11-12 min. Followed by a quick gradient change to initial conditions of 98% solvent A from 12-12.1 min. After that, an isocratic period of 98% of solvent A was kept for 12.1-14 min. The column was reconditioned with solvent B for 5 minutes before the next injection. The total analytical run time was 14 min through a linear decrease of the mobile phase.

5.2.6 Data acquisition and analysis

MassLynx and QuanLynx software's version 4.1 (Micromass, Manchester, UK) were used for data acquisition and processing. The descriptive statistics (mean, range, maximum, frequency) of the data obtained in this study were carried out using Microsoft Office Excel 2016.

5.3 Results and Discussion

5.3.1 Mycotoxin Extraction

Whilst studies have employed sample clean-up, the present study directly injected mycotoxin extracts into the LC-MS/MS without any sample clean-up. This is in line with a study on simultaneous LC/MS/MS determination of aflatoxins, fumonisins, OTA and patulin, type A & B trichothecenes, and Zearalenone, with no sample clean-up (Rudrabhatla et al., 2007). The method employed in the present study is also supported by an HPLC-ESI-MS/MS method developed to determine 33 mycotoxins in various products simultaneously. The mycotoxins were extracted with acetonitrile/water and then directly injected into an LC-MS/MS system without any clean-up (Spanjer et al., 2008). Other studies have also conducted mycotoxin analysis with no clean-up step in various matrices (Sadhasivam et al., 2017). Therefore, the extraction method efficiently isolates the targeted mycotoxins under investigation.

5.3.2 Mycotoxin Analysis

Figure 5.3: Mycotoxin Analysis using UP-ESI-LCM/MS at Stellenbosch University Central Analytical Facilities.

Mycotoxin analysis was conducted at the Stellenbosch University (CAF-LCMS Laboratory). **Figure 5.3** shows the researcher using the equipment and conducting the data analysis. Data obtained in this study on the occurrence of mycotoxins in medicinal plants are presented in **Table 5.1.** A total of 34 samples of commonly sold medicinal plants were analysed for the presence of multiple mycotoxins. None of the plant samples contained detectable levels of DON and NIV.

Table 5.1: Mycotoxin-contaminated medicinal plants.

SN	Sample	AFB_1	DON	FB_1	FB_2	FB_3	OTA	NIV	ZEN
1	MS13	-	-	**10.0**	**18.0**	**1.0**	-	-	-
2	SV06	-	-	**12.0**	**15.0**	**-**	-	-	-
31	MS11	-	-	-	-	-	-	-	**183.0**
4	MS06	-	-	-	-	-	-	-	**54.0**
5	MS04	-	-	-	-	-	**4.0**	**-**	**-**
6	MS03	-	-	-	**2.0**	-	-	-	-
7	SV13	-	-	-	-	-	-	-	**7.0**
8	SV14	-	-	-	**6.0**	-	-	-	-
9	SV03	**15.0**	-	-	-	-	-	-	-
10	SV02	-	-	**7.0**	**1.0**	**1.0**	-	-	-

Only positive sample results have been shown; Concentrations in ug/kg; **-** = Not detected

5.3.3 Frequency of Occurrence

Ten out of 34 samples (29%) were positive for mycotoxins as follows OTA (1); AFB_1 (1); FB_1 (3); FB_2 (5); FB_3 (2); ZEN (3) as illustrated on **Figure 5.3**. Multimycotoxin contamination was observed in 30 % of the positive samples with fumonisin derivatives (FB_1, FB_2, FB_3). Of the positive samples, 24% contained two or more mycotoxins. In terms of proportions, the ratio of positive samples for both *muthi* shops and street vendors was equal.

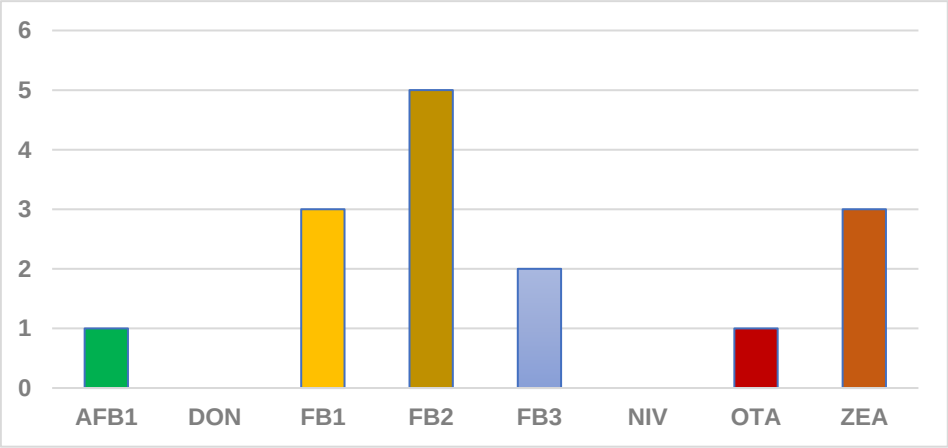

Figure 5.4: Frequency of mycotoxin occurrence in medicinal plants.

5.3.4 Mycotoxin contamination in medicinal plants

The positive samples, range, and mean for all samples analysed are presented in **Table 5.1.** The mean concentration ranged from AFB_1 (15 µg/kg), OTA (4 µg/kg), FB_1 (7-12 µg/kg) with a mean 10 µg/kg, FB_2 (1-18 µg/kg) mean 8.5 µg/kg, and FB_3 (1-15 µg/kg) mean 4.5 µg/kg with mean, ZEN (7-183 µg/kg) with a mean of 81.3 µg/kg. The highest mycotoxin contamination level in the study was recorded for ZEN at 183 µg/kg. The sample contaminated with ZEN had the highest total mycotoxin levels, whilst the least contaminated had a concentration of 1.0 µg/kg.

Table 5.2: Mycotoxin occurrence in medicinal plants.

Mycotoxin	Type of trader	Positive samples (N=10)	Range (µg/kg)	Mean (µg/kg)
AFB_1	*Muthi* Shop			
	Street Vendor	10%	15.0	15.0
OTA	*Muthi* Shop	10%	4.0	4.0
	Street Vendor			
FB_1	*Muthi* Shop	10%	10.0	10.0
	Street Vendor	20%	7.0-12.0	10.0
FB_2	*Muthi* Shop	20%	2.0-18.0	10.0
	Street Vendor	30%	1.0-15.0	7.0
FB_3	*Muthi* Shop	20%	1.0-15.0	8.0
	Street Vendor	10%	1.0	1.0
ZEN	*Muthi* Shop	30%	54.0-183.0	119.0
	Street Vendor	10%	7.0	7.0

5.3.3.1 Aflatoxin (AFB_1)

Aflatoxins have been reported as mycotoxins of human importance (Pickova et al., 2021). AFB_1 is the most predominant hepatocarcinogen and most toxic member of the aflatoxins group (Hamid et al., 2013; Marchese et al., 2018). The International Agency of Research on Cancer (IARC) has classified AFs as Group 1 human carcinogen (IARC, 1993). There are various worldwide reports on aflatoxin contamination in medicinal plants. The current study findings seem to be consistent with a report by

Aiko and Mehta (2016); in their study of 63 Indian medicinal herbs samples, only one sample tested positive for aflatoxin B. Similarly, Hu et al. (2018) found one out of 40 samples of medicinal materials positive for AFB_1. In another study of African traditional herbal medicines sold in South Africa (Tshwane-Pretoria and Cape Town), all 16 samples were not contaminated with aflatoxins (Katerere et al., 2008). A study in Italy by Romagnoli et al. (2007) found all samples of medicinal plants, aromatic herbs and herbal infusions were not contaminated with aflatoxins.

Another study of 500 herbal plants amoles in Poland reported all samples to be safe from aflatoxins. (Ledzion et al., 2011). This is similar to the current study findings, which only found one sample contaminated with AFB_1. In contrast, Mannani et al. (2020) reported aflatoxin contamination in 58.9% of herbal tea samples from the Moroccan market.

Current study findings for AFB_1 (15 µg/kg) were lower than Selim et al. (1996), who reported aflatoxin (AFB_1) contamination in 29% of the 31 samples of herbs and medicinal plants (mean 49 ppb). Tassaneeyakul et al. (2004) reported in Thailand aflatoxin contamination in herbal medicinal plant products in the range of 1.7–0.0000143 µg/kg in 5 out of 28 samples which is lower than the present findings. Furthermore, the AFB_1 concentration reported in our study was above Yang et al.'s (2005) AFs (up to 32 µg/kg) in 3 of 19 samples of Chinese herbal medicines. Commonly used Nigerian indigenous crude herbal preparations tested positive for aflatoxin contamination in the range of 0.004–0.345 µg/kg, which is also lower than the current study findings (Oyero & Oyefolu, 2009). AFB_1 was also reported in kava kava at a concentration of 0.0005 µg/kg. In the same study, other botanical root samples tested negative for aflatoxins (Weaver & Trucksess, 2010). In China, Hu et al., 2018 reported one sample of medicinal materials of radix and rhizome to be contaminated with AFB (5 µg/kg).

Aflatoxin production is affected by several factors, including fungal strain, substrate potential, oxygen supply, insect interaction and storage conditions, but temperature and relative humidity are critical (Daou et al., 2021). Optimum conditions for aflatoxin production by *A flavus* and *A parasiticus* species are at (0.94–0.99a_w) and temperatures (25–37 °C) (Lahouar et al., 2016; Gizachew et al., 2019). The climate of the Free State province, especially summer temperatures, might contribute to aflatoxin

contamination in medicinal plants. Another factor might be the climatic conditions where the plants are collected in Gauteng and KwaZulu Natal's significant markets.

The South African regulations state that all food stuffs may not contain more than 10 µg/kg of aflatoxin, and AFB_1 may not exceed 5 µg/kg. The concentration reported in this study is above the permissible limit for AFB_1 of 5 µg/kg and total AFs (10 µg/kg) (Food Stuffs and Cosmetics Disinfectant Act (Act 54 of 1972); GN R1145 (2004). The AFB_1 level reported in the study is also above the maximum limits of 2 µg/kg for AFB_1, and 4 µg/kg for total aflatoxins in herbal drugs set by the European Pharmacopoeia (EP, 2011) as well as Liu et al. (2012) proposed maximum limits of 5 µg/kg and 10 µg/kg for AFB_1 and total aflatoxins respectively. The presence of AFB_1 sheds light on the possibility of contamination of medicinal plants by aflatoxins. Therefore, consumers of medicinal plants sold in the markets might be at risk of mycotoxicosis due to aflatoxin contamination.

5.3.3.2 Deoxynivalenol (DON) and Nivalenol (NIV)

Deoxynivalenol and nivalenol are naturally occurring type B trichothecene mycotoxins produced by *Fusarium* species. DON is the most common trichothecene, followed by nivalenol, T-2 toxin and HT-2 toxin (Pinton & Oswald, 2014; Ji et al., 2019). DON and NIV were not detected in all the samples analysed in the current study. This outcome is in contrast to a study conducted in Spain which found that 62% of the 84 of 42 types of aromatic and/or medicinal herb samples analysed were contaminated with DON (Santos et al., 2009). A study of Chinese medicinal herbs and related products reported DON (17.2–50.5 µg/kg) contamination in 3 out of 58 samples (Yue et al., 2010). A most recent by Darra et al. (2019) in Lebanon on multimycotoxin occurrence in commercial spices and herbs found DON (12% in spices, 3% in herbs), but NIV was not detected.

In Latvia, deoxynivalenol was detected in 45% of marketed herbal tea samples at 129 µg/kg in the herbal blend and 5.463 µg /kg in wormwood tea (Reinholds et al., 2019). Whilst previous studies have detected the presence of DON and NIV, the absence of these mycotoxins may be attributed to the environmental conditions which do not favour the production of these mycotoxins by fungi species.

High concentrations of DON may induce vomiting, while significantly higher levels may lead to fatality. DON inhibits DNA protein synthesis and causes immunosuppression and alteration of brain neurochemicals (Petska, 2007; Bonnet et al., 2012). The European Commission has set limits of DON to 200 µg/kg for processed cereal-based food and 1250 µg/kg for unprocessed cereals (EC, 2006). In South Africa, DON is regulated for maize or barley ready for human consumption and may not contain more than 1000 µg/kg of deoxynivalenol (Food Stuffs and Cosmetics Disinfectant Act, Act 54 of 1972; GN R1145, 2004). However, regulatory limits have not been provided yet for nivalenol.

*5.3.3.3 **Fumonisins** (FB_1, FB_2, FB_3)*

Fumonisins contamination is mainly a field problem, with slightly increased levels during storage (Braun et al., 2018; Kamle et al., 2019). Fumonisins B is the common contaminant in food, with FB_1 being the most toxic form that can co-exist with other fumonisins. Other derivates, FB_2 and FB_3, which have been detected, have been shown to occur in lower concentration levels. FB_1 has been classified as a Group 2B carcinogen- 'possibly carcinogenic to humans' (IARC, 1993; Wild & Gong, 2010). In accordance with the present results, previous studies have demonstrated that medicinal plants can be contaminated with fumonisins. In Turkey, 2% of 115 medicinal plants and herbal tea samples tested positive for FB_1 at levels of 0.00016 and 0.001487 µg/kg (Omurtag & Yazicioglu, 2004).

Current study findings were lower than previous studies in South Africa. For example, Sewram et al. (2006) reported in the Eastern Cape that only 4 out of 30 medicinal wild plant samples tested were contaminated with FB_1 (8–1553 µg/kg. In another study of African traditional herbal medicines sold in Tshwane and Cape Town, 81% of 16 samples were found contaminated with FB_1 (14 –139 µg/kg) (Katerere et al., 2008). These earlier studies concluded that FB_1 contamination was more common in South African medicinal herbs, whilst the current study found FB_2 to be the predominant fumonisin derivative. Samples of black tea and medicinal plants sold from Lisbon supermarkets in Portugal tested positive (65%) for FB_1 (range, 20 to 700 µg/kg), whilst none was contaminated with FB_2 (Martins & Martins, 2001). Han et al. (2010) reported that more than 50% of 35 samples of traditional Chinese medicines tested positive for

fumonisins contamination (0.58–88.95 µg/kg). In a recent study of mycotoxin contamination in *Menthae haplocalycis*, Luo et al. (2018) reported FB_1 and FB_2 in samples analysed. The presence of fumonisins contamination has been reported before, demonstrating the risk to consumers and the need for continuous monitoring and in vitro studies in exposure risk assessments.

In South Africa, for further processing, fumonisin is regulated for raw maize grain. It may not contain more than 4000 µg/kg of FB_1 and FB_2, whereas, for maize flour, maize meal ready for human consumption has a limit of 200 µg/kg for FB_1 and FB_2 whole commodity. Present study findings for total fumonisins were below the South African regulatory limits, but this does not absolve the consumer from fumonisin mycotoxin health risks.

5.3.3.4 Ochratoxin (OTA)

The family of ochratoxins comprises three derivatives, A, B, and C. Ochratoxin A (OTA) is the family's most abundant and most toxic member. OTA mixtures or combinations with other mycotoxins may threaten human and animal health (Heussner & Bingle, 2015). Halt (1998) reported traces of ochratoxin in medicinal plant materials from *Tilia grandifolia*. In Poland, 49% of the 79 samples of herbs analysed for the natural occurrence of OTA contamination tested positive, whilst 22.3% exceeded OTA acceptable limits (Waskiewicz et al., 2013). OTA concentration from this study was higher than Roy and Kumar (1993), who reported that 44% of 129 herbal samples meant for Ayurvedic medicines were contaminated with OTA (range 0.3-0.00234 µg/kg). Aziz et al. (1998) reported higher OTA levels than our findings; in their study, 3 out of 17 medicinal plant samples were contaminated by OTA at a mean concentration of (20-80 µg/kg).

Also, Bresch et al. (2000) reported OTA contamination in 50% of the 19 liquorice samples (range, 0.3 to 216 µg/kg). A study on Chinese medicinal plants reported OTA presence in 44% of the 57 samples analysed (range 1.2-158.7 µg/kg) (Yang et al., 2010). In a recent study, Ochratoxin A (OTA) was detected in 10% of herbal teas marketed in Latvia at concentrations ranging between 2.99–30.3 µg/kg (Reinholds et al., 2019).

OTA has been classified as a possible Group 2B human carcinogen (IARC, 1993). It is reported to be associated with a fatal human kidney disease called Balkan Endemic Nephropathy, including genotoxic, hepatotoxic and other immunosuppressive properties (Pfohl-Leszkowicz et al., 2007). According to EFSA Panel on Contaminants in the Food Chain (2020), OTA has been found in breast milk, which could represent a possible health concern for breastfed infants. Shim et al. (2014) reported an OTA transfer rate of (12.72–61.33%) from herbal medicines to decoctions, indicating that using mycotoxin-contaminated medicinal plants presents a health risk to the consumers after consumption of such products. OTA is not regulated in South Africa; however, according to the European Union Commission Regulation (EC, 2006), the maximum residue level (MRL) for OTA in nutmeg, ginger, turmeric, black and white pepper, liquorice root and its extract, the legislative limit varies from 15 µg/kg to 80 µg/kg. In the current study, the OTA concentration was below the set limit as well as the European regulatory standard (5 µg/kg in unprocessed cereals) (EC, 2006).

5.3.3.5 Zearalenone (ZEN)

The mean concentration for zearalenone recorded in the current study was lower than the one reported in China by Zhang et al. (2011). All nine coix seed medicinal herb samples tested positive for ZEN (range, 18.7–211.4 µg/kg). Similarly, in another study Kong et al. (2013 also reported in China ZEN contamination of coix seeds (68.9 to 119.6 µg/kg). Whilst an earlier study by Aziz et al. (1998) of 184 medicinal plant samples did not detect ZEN in all the samples using direct determination.

Different countries have set a maximum limit for ZEN ranging from 20 to 1000 µg/kg in raw and processed food items (EC, 2006). In our study, ZEN contamination levels (7-183 µg/kg) did not exceed the permissible limits. ZEN exerts acute and systemic effects on humans. It can lead to hormonal imbalances, leading to numerous diseases such as breast cancer and prostate cancer (Rogowska et al., 2019). Advanced pubertal changes in young children have been reported in Puerto Rico and gynecomastia with testicular atrophy in rural males in Southern Africa (Milićević et al., 2010; Shepard, 2008). Oestrogenic effects in postmenopausal women were also reported as a result of using ZEN-tainted ginseng (Gray et al., 2004). The International

Agency for Research on Cancer (IARC, 1993) found ZEN to be possibly carcinogenic to humans.

5.3.3.6 Multimycotoxin contamination

In this study, mycotoxin occurrence was mainly among Fumonisin derivatives (FB_1, FB_2, FB_3). Mycotoxin co-occurrence has been reported in previous studies of medicinal plants. All 84 samples of medicinal and aromatic herbs analysed in Spain showed multi-contamination with AFs, OTA, ZEN, FBs, DON, T-2 toxin and citrinin (Santos et al., 2009). In another study, Gautam and Bhadauria (2009) reported contamination in 20.58% of the powdered herbal samples with mycotoxins (total aflatoxins, sterigmatocystin, citrinin). In an analysis of ginger products, aflatoxins and OTA were detected in 67% and 74% of samples, respectively, with a range of 1- 30 ng/g (Trucksess et al., 2007). A study by Koul and Sumbali (2008) found the presence of ZEN and DON in 13.07% and 6.92% of 130 samples of medicinally important dried rhizomes and root tubers.

Veprikova et al. (2015) analysed herbal-based dietary supplements for the presence of 57 mycotoxins. The study reported *Fusarium* trichothecenes, ZEN and ENs, and *Alternaria* as the main mycotoxins and mycotoxin co-occurrence of ENs, HT-2, T-2 and Alternaria toxins. A simultaneous analysis of multiple mycotoxins in 44 samples of *Alpinia oxyphylla* by UPLC-MS/MS detected AFB_1, ZEN, OTA, FB_1 and FB_2 in four mouldy samples (Zhao et al., 2017). Another study of multiclass mycotoxins (11) in Chinese medicinal and edible lotus seeds found that three of the ten batches of samples tested positive for AFB_1, FB_2, T-2 and ZEN. An investigation into the presence of multi-class mycotoxins in 40 batches of *Menthae haplocalycis* samples found the most common mycotoxin was tentoxin, followed by alternariol, alternariol monomethyl ether, ZEN, FB_2, FB_3, OTA, AFB_1, AFB_2, AFG_1, and T-2 toxin (Luo et al., 2018).

Reinholds et al. (2019) analysed 60 samples of herbal teas from Latvia drug stores for the presence of 12 mycotoxins. Among the dry tea samples, 90% were positive for at least one to eight mycotoxins. A study on teas and medicinal plants used to prepare infusions in Portugal reported that 84% of the analysed samples tested positive for at least one of the mycotoxins (Duarte et al., 2020). Narvaez et al. (2020), in the analysis

of the presence of 16 mycotoxins in botanical nutraceuticals, reported co-occurrence in 4 out of 10 samples (EN B1, EN A and EN A1). Although prevalent, mycotoxins were ZEN (60%), and EN B1 (30%) in the samples analysed.

A recent study by Caldeirão et al. (2021) analysed 58 herbs from Brazil for the presence of 14 mycotoxins by LC-MS/MS. Mycotoxin multiple contaminations (range 1-8) were reported in 72% of the samples. The most prevalent mycotoxins were enniatins (EN), beauvericin (BEA), sterigmatocystin (STE) and HT-2 toxin, whilst FB_1, FB_2, and T-2 were not detected in any sample. Furthermore, the concentration of mycotoxins in herbal infusion was 88% lower than in raw herbs. The percentage of positive samples and mycotoxin co-occurrence of mycotoxins varied among the different studies, which can be attributed to the sensitivity of the methods used and the wide range of mycotoxins analysed compared to the current which only investigated the presence of 8 mycotoxins. Reports from preceding studies further indicate that multiple mycotoxin contamination in medicinal plants, herbs, and herbal products is a cause of concern. Therefore, there is a need for a comprehensive analysis of other emerging mycotoxins in routine monitoring of medicinal plants and their products.

The presence of mycotoxins in the food and feed further exposes the risk of endemic diseases such as malaria, hepatitis, and HIV with consequent acute and chronic effects (Wild & Gong, 2010). The lack of epidemiological studies focusing on co-exposure to multi-class mycotoxins and associated health outcomes is partly attributable to the absence of valid biomarkers (Gong et al., 2016). A study of 53 South African women study found eight single or combined mycotoxins in urine samples, including DON, FB_1, OTA and ZEN (Shepard et al., 2013). Another study conducted in Cameroon reported detecting 11 single or combined mycotoxins and their metabolites in 63% of 175 urine samples, including AFM_1, OTA and DON (Abia et al., 2013). The presence of more than one mycotoxin demonstrates the possibility of mycotoxin exposures from single or multiple sources not limited to food but other non-food sources, as reported earlier. Therefore, the contribution of medicinal plants as a source of mycotoxin exposures should not be underestimated. Whilst co-occurrence has been reported in medicinal plants by several previous studies, the present study findings

warrant further research to analyse a wide range of mycotoxins, especially the ones not frequently studied/reported.

5.4 Conclusion

The present study evaluated the presence of mycotoxins (AFB_1, DON, FBs, NIV, OTA, and ZEN) as they have been reported to be the major mycotoxins of public health importance. The findings indicate the prevalence of mycotoxin contamination in frequently traded medicinal plants in South Africa. Mycotoxins pose a health risk to consumers due to the additive or synergistic effects of mycotoxins. Considering the frequency of use, dietary intake, and individual susceptibility, among other factors, consumers are at an increased risk of mycotoxins' adverse health effects. Current study findings supported by previous urinary biomarkers assessment reports demonstrate that medicinal plant consumers are at risk despite the low concentration levels recorded for some mycotoxins in medicinal plants and their products. This is the first study in the Free State province to investigate multiple mycotoxin contamination in marketed medicinal plants. Therefore, routine monitoring of multiple mycotoxins contamination and human exposure assessments using biomarker analysis is necessary, as well as checking the storage and trading conditions to ensure that the trade is being conducted in environments that do not favour the growth of fungi and mycotoxin production including regulation of trade.

5.5 References

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CHAPTER 6
GENERAL DISCUSSION, RECOMMENDATIONS, AND CONCLUSIONS

6.1 Introduction

The increased consumption of medicinal plants as complementary and alternative medicines has made their use a public health problem as they are susceptible to contamination by fungi and their toxins. Medicinal plants can be contaminated with several toxigenic fungi in the field during harvesting, preparation, processing, storage, and distribution (Zain, 2011). The climatic conditions of high humidity and high temperature, among other environmental conditions in Sub-Sahara, are favourable for mould growth and subsequent mycotoxin contamination (Wagacha & Muthomi, 2008). The occurrence of toxigenic fungi and mycotoxins in medicinal plants has been reported worldwide. However, studies are lacking in developing countries compared to developed countries, considering that approximately 60-80% of the black South African population relies on medicinal plant use (Nieman et al., 2019). Consequently, the economic and health impacts of fungi and mycotoxins on consumers cannot be underestimated; hence, ensuring safety and quality is vital (Ekor, 2014; Mensah et al., 2019). Despite the growth in medicinal plant trade and use, relatively few studies have been done to establish fungi and mycotoxin multi- mycotoxin contamination in South African medicinal plants (Sewram et al., 2006). Mycotoxin exposures due to intake of medicinal plants need to be assessed considering the type and quantity of medicinal plants (Altyn & Twaruzek, 2020). Furthermore, exposures may differ between different societies due to social, cultural, and economic factors, including endemic conditions in the area. Moreover, mycotoxin exposure from medicinal plants may add to dietary intake from other foods (Zain, 2011; Jai et al., 2021). Considering the daily and long-term use of medicinal plants contaminated with fungi and mycotoxins, the risk of adverse health effects increases (De Saeger & Logrieco, 2017). Therefore, the overarching aim of this study was to describe the trade in medicinal plants and determine the occurrence of mycotoxigenic fungi and mycotoxins in medicinal plants frequently sold in the Free State Province. The results from the study are summarised in **Table 6.1**.

Table 6.1: Summary of all experimental results obtained from this research project.

Objective	Method	Results
To identify the frequently traded medicinal plants in the Free State province.	Market Survey	• 165 ethno-species reported. • Trade divided into street vendors and *muthi* shops with dominance of men. • Major markets supply the province are in Johannesburg and KwaZulu Natal. • The dominant species were *Hypoxis latifolia, Dicoma anomala, Helichrysum odoratissimum, Elephantorrhiza elephantina, Tulbaghia alliacea,* and *Pentanisia prunelloides.* • Trade not regulated and conducted in areas with conditions favourable for fungi and mycotoxin contamination.
To determine the incidence of fungi contamination in commercially traded medicinal plants.	Fungal enumeration, morphological and molecular identification. Screening for fungi toxigenicity using UPLCMS/MS.	• Predominant species belonged to *Penicillium, Aspergillus, Fusarium* and *Purpureocillium*. • 26 % of the samples had no fungi contamination, 74% were contaminated by one or more fungal species. • Street vendors fungal loads ranged from 1×10^6 to 8.4×10^7; *muthi* shops fungal loads ranged from 1×10^6 to 8.4×10^7 CFU/g. • For all positive samples, the fungal load was above the WHO limits. • A total of 54 fungi isolates were recovered, comprising of 17 species.17 of the 54 fungal isolates screened for mycotoxin producing potential, 59% were toxigenic. None of the fungal isolates were able to produce NIV, OTA, and ZEN in culture.
To determine and quantify multiple mycotoxin contamination in medicinal plants.	Mycotoxin analysis using Ultra High Performance Liquid Chromatography in Tandem Mass Spectrometry.	• Ten out of 34 samples tested positive for mycotoxins as follows; AFB_1(10%); OTA (10%), FB_1 (30%), FB_2 (50%), FB_3 (20%) and ZEN (30%). • Multimycotoxin contamination was observed in 30% of the positive samples with Fumonisin derivatives (FB_1, FB_2, FB_3).

6.2 Discussion

The Free State Province's commercial trade in medicinal plants is unregulated and mainly dominated by *muthi* shops and street vendors. The demand for medicinal plants is growing, as demonstrated by the number of markets and people involved in the trade. The trade has become a source of livelihood for the unemployed and illiterate population, as reported in **Chapter 3**. The medicinal plants are mainly sourced within the province through self-collection, suppliers, Lesotho and other major markets in Johannesburg and Durban. The trade is primarily dominated by Basotho people and Zulu and Xhosa traders. Generally, traders lack awareness regarding the safety and quality of medicinal plants. The environmental conditions where the trade is conducted are also favourable for contamination by microorganisms such as fungi. Most medical plants are sold unpacked and unlabelled and with no expiry date. Since some medicinal plants are consumed daily by chronic patients or as a dietary supplement, ensuring their safety and quality is quite important (Calixto, 2000; Ekor, 2013; van Wyk & Prinsloo, 2020). Fungi species are also pathogenic and present health and economic impacts as a result of a reduction in safety, quantity, quality and efficacy of medicinal plants (Aiko & Mehta, 2016). Whist Van Vuuren et al. (2014) reported bacteriological contaminants in medicinal plants sold at the Faraday market, Johannesburg, South Africa, the contamination of medicinal plants with fungus is more prevalent than bacterial (Govender et al., 2006; Jin-Sung & Yoon, 2016; Abualhasan et al., 2019).

Approximately 71% were contaminated by more than one fungal species (**Chapter 4**)**.** The fungal load was above 1×10^6 CFU/g, indicating the prevalence of contamination in medicinal plants. Moreover, the level is above World Health Organisation's (2011) recommended limit of 1×10^3 CFU/g. The contamination of medicinal plant samples by more than one fungal species also points to the nature of health risks to consumers or deterioration in the quality of the medicinal plants. Microbial contamination can occur along the medicinal plant supply chain (Ashiq et al., 2014; Altyn & Twaruzek, 2020). Among the various fungal isolates recovered, *Penicillium, Aspergillus* and *Fusarium* were the predominant fungi which corresponded with previous studies on South African medicinal plants (Sewarm et al., 2006; Katerere et al., 2008). *Aspergillus, Penicillium*, and *Fusarium* species are the major mycotoxin producers (Alshannaq &

Yu, 2017). Therefore, their occurrence in the samples analysed further indicates a high risk of mycotoxin contamination of medicinal plants and associated adverse effects on the consumer.

Fungi contamination reported in this study based on the dominant species shows that the contamination mainly occurs post-harvest due to preservation or storage conditions. It can also be explained by the high temperature and humidity experienced in KZN and Gauteng markets where the plants are sourced as well as collection areas and the climatic conditions of the Free State province. Another factor observed during the survey is the use of open plastic bags and maize sacs packaging whilst displaying the medicinal plants might also contribute to the fungi contamination.

Other species isolated include *Phoma*, *Purpureocillium* and *Epicoccum*. *Phoma* are mainly plant pathogens and act as opportunistic pathogens in susceptible individuals (Bennet et al., 2018). Due to their widespread geographical distribution and their presence in analysed samples indicates contamination of medicinal plants due to environmental factors. Furthermore, it points to a possibility of the *Phoma* species having infected the plant before harvesting (Deb et al., 2020). *Epicoccum* species are found worldwide in the environment where they interact with the plant and other endophytes and pathogens. They produce a wide range *of* secondary metabolites, making them valuable in biomedicine and agriculture. However, it is a common allergen but rarely causes systemic diseases in humans (Flannagan et al., 2002; Bragga et al., 2018;). *Purpureocillium* is a common fungus found in soil, insects, and decaying matter. It is used as a biocontrol agent in agriculture and biomedicine, although it has been reported to cause ocular mycosis in immunocompromised individuals (Luangsa-Ard et al., 2011; Chew et al., 2016). Whilst human infections due to *Purpureocillium species* are rare. Still, the presence in analysed samples is also a cause for concern to protect patients who use medicinal plants as natural drugs.

All fungal isolates did not produce NIV, OTA and ZEN in culture. Although *Penicillium, Aspergillus*, and *Fusarium* are the major producers of mycotoxins, not all species are toxigenic (Perrone et al., 2020). As reported from this study, 74% of the samples were contaminated by fungi. Also, screening for mycotoxin-producing potential of isolates showed that some known toxigenic species could not produce mycotoxins in culture. This can be attributed to the various factors that promote mycotoxin production, which

was not optimised in the current study as culturing was done under average room temperatures and conditions. Furthermore, mycotoxin production is also influenced by train specificity, variation, and interactions between toxigenic fungal species (Pitt et al., 2000; Greeff-Laubscher et al., 2018; Awuchi et al., 2021).

In the current study, none of the plant samples contained detectable levels of deoxynivalenol and nivalenol. Approximately 29% of the samples were contaminated with mycotoxins, while 30% of the positive samples showed multiple contaminations with Fumonisins (B_1, B_2, B_3). Moreover, some samples which tested positive for mycotoxins did not have fungal contamination with toxigenic species indicating that the absence of fungi contamination does not mean the absence of mycotoxins. It is interesting to note that none of the medicinal plants was positive for DON, but some isolated species were able to produce DON on synthetic media.

Furthermore, medicinal plant samples tested positive for OTA and ZEN, but none of the isolated fungal species produced detectable amounts of these toxins. This supports previous studies, which also showed that the presence of toxigenic fungi does not necessarily imply the presence of mycotoxins. Some samples from which toxigenic fungi were isolated also tested negative for natural mycotoxin contamination.

An earlier study by Abou-Arab et al. (1999) reported the occurrence of *Aspergillus*, *Penicillium*, and *Fusarium* species in medicinal plants; however, natural mycotoxin contamination was absent in all the samples. Aiko and Mehta (2016) also reported the absence of mycotoxins despite having isolated toxigenic fungi from medicinal herbs and spices. In agreement with the findings of earlier studies, we also suggest that presence of toxigenic fungi is not an indication of mycotoxin contamination in medicinal plants. A possible explanation for these results may be that the matrix effects of medicinal plants are not suitable for toxin production, since some fungal strains produced toxins in synthetic medium, but their source samples tested negative for mycotoxin contamination. The absence of mycotoxin contamination in samples may also be due to the inherent anti-mycotoxin properties of medicinal plants such as essential oils (Sumalan et al., 2013; Prakash et al., 2014). There are several reports on the use of medicinal plants as antifungal and anti-mycotoxin agents (Hassan & Ullah, 2019; Samadi et al., 2019; Makhuvel et al., 2020; Mishra et al., 2020). We can also postulate that the absence of visible moulds does not correspond to the lack of

mycotoxins contamination, hence the need for mycotoxin sampling for all types of herbal raw materials sold in markets irrespective of physical appearance.

6.3 Limitations of the study

Time limitations regarding completion of master's project and thesis submission coupled with the SARS-CoV-2/ COVID-19 pandemic prevented a detailed investigation into the medicinal plant trade and further assessment of preservation and storage methods and hygienic practices of the various players involved in the medicinal plant trade chain. The study was limited only to street vendors and *muthi* shops and excluded other traditional healers and herbalists who do not sell directly to the public. The findings are limited only to the study areas and may not apply to other areas outside the Free State province.

6.4 Areas for Future Research

a. Further studies to associate multiple mycotoxin exposure and human health, possibly via identification and validation of suitable biomarkers to assess their risks to medicinal plant consumer's health in South Africa.
b. Evaluation of the effectiveness of the various storage methods used in medicinal plant preservation starting from the collectors up to the traders.
c. Studies including suppliers, wholesalers, and traditional healers in mycotoxin analysis of medicinal plants to determine points within the supply chain where fungal species and mycotoxins contaminate the medicinal plants.
d. More research on interactions between mixed exposure (dietary intakes and medicinal plant use) to draw factual conclusions on the health impact of human exposure to the mycotoxin.
e. Isolation and identification of plant fungal endophytic strains to be tested for their ability to produce disease symptoms or whether they protect the plant against pathogenic isolates or give it medicinal properties.

6.5 Recommendations

a. Medicinal plant trade to be regulated, and local governments to ensure proper and standard trading facilities are used.
b. Standards for maximum fungi and mycotoxin levels are to be stipulated for South African medicinal plants and their products.
c. Prevention and control measures focused on capacitating the various medicinal plant trade-chain players with skills and knowledge related to GAP, GMP, and storage practices.
d. Creation of formal enterprises dealing in medicinal plants to ensure that safe, good quality, packaged medicinal products are sold to the public.
e. In-depth monitoring to assess public health risk and implementation strategies to reduce consumer exposure to mycotoxins.
f. Routine mycotoxin analysis of commercial plant materials before use to ensure they are safe for consumers.
g. Public health promotion campaigns to increase awareness of mycotoxins in medicinal plants and products not regulated by the government.

6.6 Conclusions

The current study provides information on the occurrence of fungi and mycotoxins in medicinal plants sold in the Free State, South Africa. Contamination of these products with toxigenic fungi poses serious health threats since their presence can cause ill effects rather than improve the quality of life. This study concludes that medicinal plants are susceptible to contamination by toxigenic fungi and mycotoxins. It has been shown that commercially available medicinal plants can be contaminated with mycotoxins at levels exceeding South African regulations and other standards. Proper storage conditions and quality control are essential at every stage of processing, packaging, or marketing for the consumers' safety. Maximum limits of common mycotoxins should be officially specified for various medicinal plants and their products. Therefore, strategies to mitigate fungi and mycotoxin contamination must be enacted at significant markets, traditional healers, local suppliers, *muthi* shops and street vendors.

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Appendices

a. Health Sciences Research and Ethics Committee Approval.

UNIVERSITY OF THE FREE STATE
UNIVERSITEIT VAN DIE VRYSTAAT
YUNIVESITHI YA FREISTATA

UFS·UV
HEALTH SCIENCES
GESONDHEIDSWETENSKAPPE

Health Sciences Research Ethics Committee

04-May-2020

Dear **Mr Julius Ndoro** Ethics Clearance: **Mycotoxigenic fungi and multimycotoxin contamination in medicinal plants, Mangaung South Africa**

Principal Investigator: **Mr Julius Ndoro**

Department: **Environmental Health Sciences - CUT APPLICATION APPROVED**

Please ensure that you read the whole document

With reference to your application for ethical clearance with the Faculty of Health Sciences, I am pleased to inform you on behalf of the Health Sciences Research Ethics Committee that you have been granted ethical clearance for your project.

Your ethical clearance number, to be used in all correspondence is:**UFS-HSD2019/1226/2605**

The ethical clearance number is valid for research conducted for one year from issuance. Should you require more time to complete this research, please apply for an extension.

We request that any changes that may take place during the course of your research project be submitted to the HSREC for approval to ensure we are kept up to date with your progress and any ethical implications that may arise. This includes any serious adverse events and/or termination of the study.

A progress report should be submitted within one year of approval, and annually for long term studies. A final report should be submitted at the completion of the study.

The HSREC functions in compliance with, but not limited to, the following documents and guidelines: The SA National Health Act. No. 61 of 2003; Ethics in Health Research: Principles, Structures and Processes (2015); SA GCP(2006); Declaration of Helsinki; The Belmont Report; The US Office of Human Research Protections 45 CFR 461 (for non-exempt research with human participants conducted or supported by the US Department of Health and Human Services- (HHS), 21 CFR 50, 21 CFR 56; CIOMS; ICH-GCP-E6 Sections 1-4; The International Conference on Harmonization and Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH Tripartite), Guidelines of the SA Medicines Control Council as well as Laws and Regulations with regard to the Control of Medicines, Constitution of the HSREC of the Faculty of Health Sciences.

For any questions or concerns, please feel free to contact HSREC Administration: 051-4017794/5 or email EthicsFHS@ufs.ac.za.

Thank you for submitting this proposal for ethical clearance and we wish you every success with your research.

Yours Sincerely

Dr. SM Le Grange
Chair : Health Sciences Research Ethics Committee

Health Sciences Research Ethics Committee
Office of the Dean: Health Sciences
T: +27 (0)51 401 7795/7794 | E: ethicsfhs@ufs.ac.za
IRB 00011992; REC 230408-011; IORG 0010096; FWA 00027947
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www.ufs.ac.za

b. Environment and Biosafety Ethics Committee Approval.

UNIVERSITY OF THE FREE STATE
UNIVERSITEIT VAN DIE VRYSTAAT
YUNIVESITHI YA FREISTATA

UFS·UV
RESEARCH DEVELOPMENT
NAVORSINGSONTWIKKELING

Environmental & Biosafety Research Ethics Committee

15-Apr-2020

Dear **Mr Julius Ndoro**

Project Title: **Mycotoxigenic fungi and multimycotoxin contamination in medicinal plants in Mangaung South Africa**

Department: **Environmental Health Sciences - CUT**

APPLICATION APPROVED

This letter confirms that this research proposal was given ethical clearance by the Biosafety & Environmental Research Ethics Committee of the University of the Free State.

Your ethical clearance number, to be used in all correspondence is **UFS-ESD2019/0183/1504**

Please note the following:

1. **This ethical clearance is valid for one year from the issuance of this letter.**
2. **If the research takes longer than one year to complete, please submit a Continuation Report to the Ethics Committee before ethical clearance expires.**
3. **If any changes are made during the research process (including a change in investigators), please inform the Ethics Committee by submitting an Amendment.**
4. **When the research is concluded, please submit a Final Report to the Ethics Committee.**

Thank you for your application and we wish you well in all of your research endeavours.

Yours Sincerely

Prof. RR (Robert) Bragg
Chairperson: Biosafety & Environmental Research Ethics Committee
University of the Free State

Directorate: Research Development
T: +27 (0)51 401 9398 | +27 (0)51 401 2075 | E: smitham@ufs.ac.za
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205 Nelson Mandela Drive | Park West, Bloemfontein 9301 | South Africa
P.O. Box 339 | Bloemfontein 9300 | South Africa | www.ufs.ac.za

c. Informed Consent Form

Mycotoxigenic fungi and multimycotoxin contamination in medicinal plants, Free State province of South Africa.

Informed Consent Form for traditional healers, herbalists, commercial medicinal plant suppliers, medicinal plant traders, other people involved in the collection and trade of medicinal plants in the Free State Province.

Name of Principle Investigator: Julius Ndoro

Name of Organisation: Central University of Technology Free State

Name of Sponsor: CUT & NRF

Ethical clearance Number:

This Informed Consent Form has two parts:

1. Information Sheet (to share information about the study with you)
2. Certificate of Consent to participate in this study (for signatures if you choose to participate)

You will be given a copy of the full Informed Consent Form

Part I: Participant Information Sheet

Introduction

I am Julius Ndoro, studying at the Central University of Technology, Bloemfontein Campus. I am doing research on the trade of medicinal plants which are mostly common in this area. I am going to give you information and invite you to be part in this research. You do not have to decide today whether, or not you will participate in the research. Before you decide, you can talk to anyone you feel comfortable with about the research.

This consent form may contain words that you do not understand. Please ask me to stop as we go through the information, and I will take time to explain. If you have questions later, you can ask me or of another researcher.)

Purpose of the research

Medicinal plants are used by many people in your community, and we don't know the way they are harvested, prepared, stored and if they are safe for use by consumers. We want to explain their trade, methods of preparation and storage and determine their safety from germs. We believe that you can help us by telling us what you know both about medicinal plant uses and trade in your area.

Type of Research Intervention

This research will involve your participation in a semi structured interview that will take about one hour.

Participant Selection

You are being invited to take part in this research because we feel that your experience in the use and trade of medicinal plants can contribute much to our understanding and knowledge of the trade in medicinal plants in this area.

Voluntary Participation

Your participation in this research is entirely voluntary. It is your choice whether to participate or not. If you choose not to participate there are no consequences. You may change your mind later and stop participating even if you agreed earlier.

Procedures

We are asking you to help us learn more about medicinal plant uses trade in your community. We are inviting you to take part in this research project. If you accept, you will be asked to answer questions which will be recorded on the questionnaire during an interview. Questions range from but not limited to, education status, nature of business, where you collect your plants common medicinal plants names, preparation and uses/

You will not be asked to share personal beliefs, practices or stories and you do not have to share any knowledge that you are not comfortable sharing.

During the interview, I or another interviewer will sit down with you in a comfortable at the place of your choice. If you do not wish to answer any of the questions during the interview, you may say so and the interviewer will move on to the next question. No one else but the interviewer will be present unless you would like someone else to be there. The entire interview will be digitally recorded, but no-one will be identified by name on the recording. The data will be stored on the researcher's computer and

password protected and backed up on One Drive. The information recorded is confidential, and no one else except Mr. J Ndoro and Dr Manduna will have access to the recordings.

Duration

The research takes place over 12 months in total. During that time, we may visit you at least three times for interviewing you at different intervals and first interview will last for about one hour and subsequently for less than an hour.

Risks

We are asking you to share with us some very personal and confidential information, and you may feel uncomfortable talking about some of the topics. You do not have to answer any question or take part in the interview if you don't wish to do so, and that is also fine. You do not have to give us any reason for not responding to any question, or for refusing to take part in the interview.

Confidentiality

The research being done in the community may draw attention and if you participate you may be asked questions by other people in the community. We will not be sharing information about you to anyone outside of the research team. The information that we collect from this research project will be kept private. Any information about you will have a number on it instead of your name. Only the researchers will know what your number is, and we will lock that information up with a lock and key. It will not be shared with or given to anyone except the principal investigator, Central University of Technology and National Research Foundation.

Sharing the Results

Nothing that you tell us today will be shared with anybody outside the research team, and nothing will be attributed to you by name. The knowledge that we get from this research will be shared with you and your community before it is made widely available to the public. Each participant will receive a summary of the results. There will also be small meetings in the community, and these will be announced. Following the meetings, we will publish the results so that other interested people may learn from the research.

Right to Refuse or Withdraw

You do not have to take part in this research if you do not wish to do so and choosing to participate will not affect your practice or business in any way. You may stop participating in the interview at any time without any consequences. I will give you an opportunity at the end of the interview to review your remarks, and you can ask to modify or remove portions of those, if you do not agree with my notes or if I did not understand you correctly.

Who to Contact?

If you have any questions, you can ask them now or later. If you wish to ask questions later, you may contact any of the following: ***Julius Ndoro on _____----.*** This proposal has been reviewed and approved by *Human Research and Ethics Committee.*

You can ask me any more questions about any part of the research study, if you wish to. Do you have any questions?

Part II: Certificate of Consent

- I have read the foregoing information, or it has been read to me. I have had the opportunity to ask questions about it and any questions I have been asked have been answered to my satisfaction. I consent voluntarily to be a participant in this study.
- I…… voluntarily agree to participate in this research study.
- I understand that even if I agree to participate now, I can withdraw at any time or refuse to answer any question without any consequences of any kind.
- I understand that I can withdraw permission to use data from my interview within two weeks after the interview, in which case the material will be deleted.
- I have had the purpose and nature of the study explained to me in writing and I have had the opportunity to ask questions about the study.
- I understand that I will not benefit directly from participating in this research.
- I agree to my interview being audio-recorded.

- I understand that all information I provide for this study will be treated confidentially.
- I understand that in any report on the results of this research my identity will remain anonymous.
- I understand that disguised extracts from my interview may be quoted in publications, conference presentations, magazine articles.
- I understand that if I inform the researcher that myself or someone else is at risk of harm, they may have to report this to the relevant authorities - they will discuss this with me first but may be required to report with or without my permission.
- I understand that signed consent forms and original audio recordings will be retained at CUT and only accessible to Julius Ndoro and Dr Manduna for at least 5 years.
- I understand that a transcript of my interview in which all identifying information has been removed will be retained for five years.
- I understand that under freedom of information legalisation I am entitled to access the information I have provided at any time while it is in storage as specified above.
- I understand that I am free to contact any of the people involved in the research to seek further clarification and information.

Print Name of Participant_____

Signature of Participant _____

Date _____

If illiterate

I have witnessed the accurate reading of the consent form to the potential participant, and the individual has had the opportunity to ask questions. I confirm that the individual has given consent freely.

Print name of witness_____ Thumb print of participant

Signature of witness _____

Date _____

Statement by the researcher/person taking consent.

I have accurately read out the information sheet to the potential participant, and to the best of my ability made sure that the participant understands the purpose of the research study.

I confirm that the participant was given an opportunity to ask questions about the study, and all the questions asked by the participant have been answered correctly and to the best of my ability. I confirm that the individual has not been coerced into giving consent, and the consent has been given freely and voluntarily.

A copy of this ICF has been provided to the participant.

Print Name of Researcher/person taking the consent_____

Signature of Researcher /person taking the consent_____

Date _____

d. Questionnaire: Interview Guide

An investigation into the trade of medicinal plants in the Free State Province, South Africa.

No…….

Market Survey Interview Guide

- Interviewer's name: ……
- Date of interview: ……
- Was an interpreter used? ……
- Name of Interpreter……
- Where was the interview conducted? ……

1. Location

a. Town……

b. Village……
c. Urban/Rural……
d. Is this area your permanent residence ……
e. Contact Details……
f. GPRS Coordinates……

2. Personal Information

a. What is your Name……
b. Sex: ……
c. Age: ……
d. Race……
e. Ethnic group: ……
f. Level of education: ……
g. What is your marital status……
h. Contact details……
……

3. Business information

a. What is the nature of your business? (vendor, market, herbalist, *muthi* shop, traditional healer) ……

a. Why are you involved in the business?
……
……
……

b. Are you in the business full-time or part-time?

.....
.....

c. Do you have other sources of income?
.....
.....
.....

d. How long have you been in business? ……..

e. What is your monthly income from selling medicinal plants?

……

f. Who are your main customers?

……

……

g. How many customers do you serve per day (include gender, ages, tribes, ethnicity and occupations where relevant)?

……

……

……,……

h. Which plants do you sell the most or that you make sure you always have in stcok……

……

……

……

4. **Knowledge and Collection of medicinal plants**

a. Do you have any knowledge of medicinal plants?

……

……

b. How did you get your knowledge of medicinal plants?

……

……..

c. How do you know where the plants grow?

……

……

……

d. Where do you get the medicinal plants for selling? (Name of supplier or market)

……

……

……

e. Do you collect the plants yourself?

.....

.....

.....

f. If not, who collects/ supplies you?

.....

.....

a. Where are your plants collected?

.....

.....

b. How far is the collection site(s) /markets for most of your plants?

……

……

c. Do you collect most of your medicinal plants dry or fresh

……

……

Preservation and Storage

a. If fresh, how do you dry them?

……

……

……

……

……

b. How do you prepare for storage of your newly collected plants?

.....

.....

.....

.....

.

c. How do you package your medicinal plants for storage?

.....

.....

.....

.

d. How do you store dried medicinal plants or fresh ones?

.....

.....

e. How do you safeguard your stock from insects and contamination?

.....

.....

.....

f. How long do you keep plant materials before they go bad or lose their healing properties?

.....

.....

g. How do you recognise that a medicinal plant material is bad or spoiled?

.....

.....

h. In your own opinion what do you think causes spoilage of plant materials?

.....

.....

i. What do you do to the spoiled plant materials?

.....

.....

.

j. Do you throw medicinal plants away after losing their healing properties?

.....

.....

Comments

.....

.....

e. List of medicinal plants commonly old in the Free State province South Africa

1. African Potato
2. Amathunzandaba
3. Bobatsi
4. Boka
5. Bolao
6. Bophipi
7. Buliba
8. Cheche
9. Dabula
10. Dirahidibone
11. Hamhla
12. Hlaba
13. Hlampumpu
14. Hlokoana Tsela
15. Hlomani
16. Impikayiboni
17. Ingola
18. Inhlaba
19. Kalimuzi
20. Kathanywu
21. Khaba
22. Khahla
23. Khamane
24. Kherere
25. Khoara
26. Khonathi
27. Khopa
28. Khutsana
29. Kogwane
30. Labatheka
31. Lehe La Mqshe
32. Lehlatso
33. Lekhalaba
34. Lelemekhomo
35. Lematla
36. Lematlana
37. Lengana
38. Lensikitlane
39. Lephelephele
40. Leraratau
41. Leshomokho
42. Letapiso
43. Lethlitshani
44. Lethomoko
45. Letjoetsane
46. Letutla
47. Mabona
48. Mabophe
49. Mabutsetsa
50. Mahlanganisa
51. Majakabone
52. Makhulela
53. Maleleka
54. Mapipha
55. Marakalla
56. Mashoa
57. Mathethebala
58. Mathethebani
59. Mathuma
60. Mathunga
61. Mavhulakuvhaliwe
62. Mavhumbuka
63. Memeza
64. Metsimatso
65. Mlafeni
66. Mlomo Monate
67. Mlulama
68. Mnyisa
69. Moelewa
70. Mofetola
71. Mofif
72. Mohlatso
73. Mohloare
74. Moithimolo
75. Moisapelo
76. Mokata
77. Mokubetso
78. Monatja
79. Monna Motso
80. Moomang
81. Mopesthe
82. Morakalla
83. Morarathaba
84. Moretele
85. Moretseng
86. Morietsani
87. Mororo Wa Lipela
88. Mosikanokana
89. Mosa Pelo
90. Mosisili
91. Mosisona
92. Mositsane
93. Mothokho
94. Mototse
95. Mpahalamelo
96. Mpakanyelo
97. Mpinda
98. Msebele
99. Mthuku
100. Mudhubhuza
101. Mukhanyakhuti
102. Mukhutilu
103. Muso Pelo
104. Nkokophili
105. Ngomasemqondo
106. Ngudhuza
107. Ngwavhuma
108. Nhlanhlamlope
109. Ntelezi
110. Nyathela
111. Pelolidamolawa
112. Phefo
113. Phila
114. Phindemva
115. Phoa
116. Phonyoka
117. Pinda
118. Qobo
119. Qombo
120. Ralikokotoane
121. Ramqweni
122. Rosilina
123. Sahlulamanye
124. Seboka
125. Sefosho
126. Seherane
127. Seipone
128. Selepe
129. Seletjane
130. Selomi
131. Senyarela
132. Sepokla
133. Seqakashi
134. Serobe
135. Sesepa Sa Linoha
136. Sesumo
137. Setea Tea
138. Sethoto
139. Setimamollo
140. Sibhama
141. Sibuke
142. Sihamela
143. Sihlenhle
144. Sihletile
145. Sikhumbili
146. Sikhundhla
147. Siphaka
148. Siphephetho
149. Sithumo
150. Sithuthu
151. Tabola
152. Thola
153. Tholana
154. Thupaputswe
155. Tsebepehla
156. Tsehlo
157. Tsejananyana
158. Tsoene
159. Tsukumbili
160. Tsupane
161. Tswetlane
162. Tswini
163. Unukani
164. Upupuku
165. Vum

f. Study Photographs

Photograph 1: Street vendor displaying medicinal plants in maize bags sacs.

Photograph 2: Aggregated Street vendors.

Photograph 3: Street vendors in a linear pattern along a pavement.

Photograph 4: Street vendors displaying medicinal plants in plastic bags.

g. Published article.

Ndoro, J., Manduna, I. T., Nyoni, M., and de Smidt, O. 2022. "Multiple Mycotoxin Contamination in Medicinal Plants Frequently Sold in the Free State Province, South Africa Detected Using UPLC-ESI-MS/MS." *Toxins*. 14(10): 690. https://doi.org/10.3390/toxins14100690.

toxins

MDPI

Communication

Multiple Mycotoxin Contamination in Medicinal Plants Frequently Sold in the Free State Province, South Africa Detected Using UPLC-ESI-MS/MS

Julius Ndoro [1], Idah Tichaidza Manduna [2,*], Makomborero Nyoni [3] and Olga de Smidt [2]

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[2] Centre for Applied Food Sustainability and Biotechnology (CAFSaB), Central University of Technology, Free State, Bloemfontein 9300, South Africa
[3] Research, Development and Innovation Department, National Biotechnology Authority, 21 Princess Drive Newlands, Harare, Zimbabwe
* Correspondence: imanduna@cut.ac.za

Abstract: Medicinal plants are important in the South African traditional healthcare system, the growth in the consumption has led to increase in trade through *muthi* shops and street vendors. Medicinal plants are prone to contamination with fungi and their mycotoxins. The study investigated multiple mycotoxin contamination using Ultra High Pressure Liquid Chromatography–Tandem Mass Spectrometry (UPLC-ESI-MS/MS) for the simultaneous detection of Aflatoxin B1 (AFB1), Deoxynivalenol (DON), Fumonisins (FB_1, FB_2, FB_3), Nivalenol (NIV), Ochratoxin A (OTA) and Zearalenone (ZEN) in frequently sold medicinal plants. Medicinal plant samples (n = 34) were purchased and analyzed for the presence of eight mycotoxins. DON and NIV were not detected in all samples analyzed. Ten out of thirty-four samples tested positive for mycotoxins —AFB_1 (10.0%); OTA (10.0%); FB1 (30.0%); FB2 (50.0%); FB3 (20.0%); and ZEN (30.0%). Mean concentration levels ranged from AFB_1 (15 µg/kg), OTA (4 µg/kg), FB_1 (7–12 µg/kg), FB_2 (1–18 µg/kg), FB_3 (1–15 µg/kg) and ZEN (7–183 µg/kg). Multiple mycotoxin contamination was observed in 30% of the positive samples with fumonisins. The concentration of AFB_1 reported in this study is above the permissible limit for AFB1 (5 µg/kg). Fumonisin concentration did not exceed the limits set for raw maize grain (4000 µg/kg of FB_1 and FB_2). ZEN and OTA are not regulated in South Africa. The findings indicate the prevalence of mycotoxin contamination in frequently traded medicinal plants that poses a health risk to consumers. There is therefore a need for routine monitoring of multiple mycotoxin contamination, human exposure assessments using biomarker analysis and establishment of regulations and standards.

Keywords: medicinal plants; mycotoxins; Ultra High Pressure Liquid Chromatography–Tandem Mass Spectrometry; contamination; street vendors; *muthi* shops; fungi; plant trade

Key Contribution: This is the first report on multiple mycotoxin contamination in Free State Province, South Africa in marketed medicinal plants using UPLC-ESI-MS/MS. The study shows the contribution of commercially traded medicinal plants in human mycotoxin exposure. The study findings help in advocating for consumer safety and quality monitoring as well as development of strategies to maintain the safety of medicinal plants sold in markets.

Citation: Ndoro, J.; Manduna, I.T.; Nyoni, M.; de Smidt, O. Multiple Mycotoxin Contamination in Medicinal Plants Frequently Sold in the Free State Province, South Africa Detected Using UPLC-ESI-MS/MS. *Toxins* **2022**, *14*, 690. https://doi.org/10.3390/toxins14100690

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1. Introduction

There has been a steady increase in the demand for medicinal plants, herbs and preparations as complementary and alternative medicine (CAM) and in traditional medicine both in developing and developed countries [1]. In developed countries, between 25 and 70% of the population rely on complementary and/or alternative medicine (CAM) [2]. In

Africa, the use of medicinal plants and consumption is significantly higher, around 80%, due to economic, social, and cultural factors. Medicinal plants play a vital role in disease prevention and their promotion and use compliments current prevention strategies under the Primary Health Care Approach [3,4].

Due to a complex supply chain involving different players and conditions from pre-harvesting, harvesting, storage and trade, medicinal plants are prone to infestation by pests, microbes and toxins [5,6]. Mycotoxins are toxic fungal secondary metabolites and are common contaminants of both human food and animal feed. Contamination is more common in developing countries with poor crop storage and production technologies, and climatic conditions which promote fungal growth and toxin production [7]. There are over 400 mycotoxins known today. Aflatoxins, ochratoxins, fumonisins and trichothecenes are the major classes of mycotoxins that have been recognized as being of public health significance due to their high occurrence and associated carcinogenic properties [6,8].

Mycotoxin exposures occur via various routes of entry such as oral, dermal, respiratory and parenteral. The oral/ingestion route is the major route of entry for mycotoxin exposures. A potential chain reaction can occur when contaminated animal feed results in infected meat, milk and eggs [9] which in turn, can affect human health. Acute and chronic mycotoxicosis can be developed depending on an individual's susceptibility, the type of mycotoxin and dosage [7,10]. For example, approximately a third of all cases of liver cancer in Africa are due to chronic exposures to mycotoxins [11]. Table 1 shows the adverse effects of some mycotoxins on animal and human health [9,12–15]. Additive or synergistic harmful effects may also be a result of co-occurring mycotoxins [12]

Table 1. Mycotoxigenic effects on animal and human health.

Mycotoxin	Fungal Source (Genus)	Health Effects
Aflatoxin B1	*Aspergillus*	Teratogenic, hepatotoxic, immunosuppressive, carcinogenic and mutagenic.
Deoxynivalenol	*Fusarium*	Gastrointestinal damage, reproductive effects toxicosis, genotoxicity and immunosuppressive.
Fumonisins	*Fusarium*	Teratogenic, carcinogenic, hepatotoxic, nephrotoxic, immunosuppressive and neurotoxic.
Nivalenol	*Fusarium*	Anorexic, immunotoxic, hematotoxic and genotoxic.
Ochratoxin A	*Aspergillus Penicillium*	Carcinogenic, teratogenic, immunosuppressive and nephrotoxic.
Zearalenone	*Fusarium*	Carcinogenic, hormonal imbalance (hyperestrogenism) and reproductive effects.

Despite the reported and potential impacts of mycotoxins including their relation to many diseases, they are poorly studied in South African medicinal plants sold in markets which are prone to contamination [16,17]. Furthermore, the control of mycotoxins is inadequately funded, and many African governments do not give priority to mycotoxin control in medicinal plants [18]. However, the occurrence of mycotoxins has been reported in South Africa [16,17]; Kenya [19–21]; Nigeria [22–25]; and Egypt [26–29].

Most of these studies have been limited in scope focusing mainly on aflatoxin and fumonisin contamination. In view of the increasing demand for and trade in medicinal plants and the health risks from fungal contamination and their toxins, there is a need to have a broad understanding of the prevalence of mycotoxins in commercially traded medicinal plants. Regrettably, there is limited information on mycotoxins in medicinal plants in South Africa which is not commensurate with the escalating economic value of the trade. As mentioned earlier, previous studies have been completed in South Africa, but no studies have been published on mycotoxin contamination in medicinal plants sold in the Free State Province *muthi* (traditional medicine) shops and by street vendors, hence there is

no information available. The aim of this study was to assess the safety of medicinal plants with respect to multiple mycotoxin contamination namely Aflatoxin B_1 (AFB_1), Ochratoxin A (OTA), Zearalenone (ZEN), Deoxynivalenol (DON), Nivalenol (NIV) and Fumonisins (FB_1, FB_2, FB_3) as supported by Keter et al. [20].

2. Results

2.1. Mycotoxin Extraction

Whilst other studies have employed sample clean up, the present study directly injected mycotoxin extracts into the LC-MS/MS without any simple clean up. This is in line with a study on simultaneous LC/MS/MS determination of aflatoxins, fumonisins, OTA and patulin, type A and B trichothecenes and Zearalenone, with no sample clean-up [30] The method employed in this study is also supported by an HPLC-ESI-MS/MS method which was developed for simultaneous determination of 33 mycotoxins in various products. The mycotoxins were extracted with acetonitrile/water and then directly injected into a LC-MS/MS system without any clean-up [31]. Other studies have also conducted mycotoxin analysis with no clean-up step in various matrices [32]. Therefore, the extraction method was quite efficient in isolating the targeted mycotoxins under investigation.

2.2. Mycotoxin Analysis

A total of 34 samples from commonly sold medicinal plants were analyzed for the presence of multiple mycotoxins. None of the plant samples contained detectable levels of DON and NIV. Of the 34 samples, 10 (29%) were positive for OTA (1); AFB_1 (1); FB_1(3); FB_2(5); FB_3 (2); and ZEN (3) as illustrated in Figure 1. Multi-mycotoxin contamination was observed in 30% of the positive samples with fumonisin derivatives (FB_1, FB_2, FB_3).

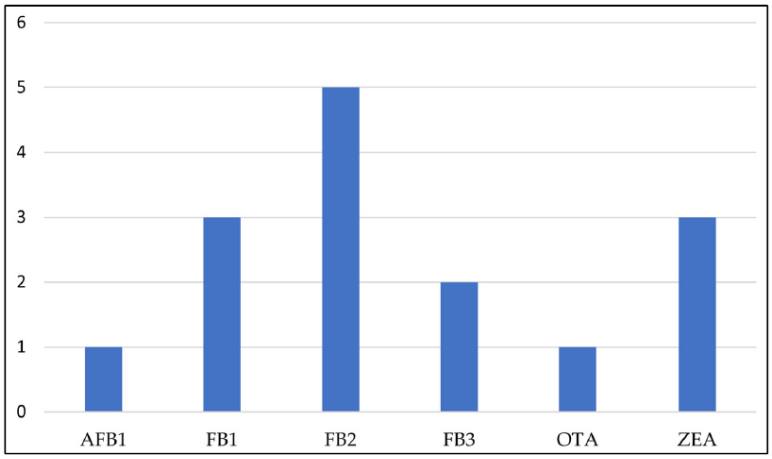

Figure 1. Frequency of occurrence of mycotoxins in medicinal plants.

The occurrence of mycotoxins and their concentrations in medicinal plants are presented in Table 2. AFB1 was only found in *Dicoma anomala* at a concentration of 15 µg/kg and OTA (4 µg/kg) was found in *Aloe ferox*. FB1 ranged from 1 µg/kg to 12 µg/kg while FB_2 was detected in five different pants from five different locations. FB_2 concentrations ranged between 1 µg/kg and 18 µg/kg. FB_3 (1–15 µg/kg) with a mean of 4.5 µg/kg and ZEN (7–183 µg/kg) with a mean of 81.3 µg/kg. The highest mycotoxin contamination level in the study was recorded for ZEN at 183 µg/kg. A sample contaminated with ZEN had the highest total mycotoxin levels whilst the least contaminated had a concentration of 15 µg/kg.

Table 2. Mycotoxin contamination in medicinal plants.

Plant Name	Trader: Location	AFB_1	FB_1	FB_2	FB_3	OTA	ZEN
Bulbine narcissifolia Salm-Dyck	MS: Thaba 'Nchu	-	10.0	18.0	1.0	-	-
Helichrysum odoratissimum (L.) Sweet.	SV: Zastron	-	12.0	15.0	-	-	-
Hypoxis hemerocallidea Fisch., C.A.Mey. & Avé-Lall.	MS: Dewetsdorp	-	-	-	-	-	183.0
Adenia gummifera (Harv.) Harms	MS: Sasolburg	-	-	-	-	-	54.0
Aloe ferox Mill.	MS: Senekal	-	-	-	-	4.0	-
Galium capense Thunb	MS: Winburg	-	-	2.0	-	-	-
Siphonochilus aethiopicus (Schweif.) B.L. Burt	SV: Kroonstad	-	-	-	-	-	7.0
Helichrysum odoratissimum (L.) Sweet.	SV: Kroonstad	-	-	6.0	-	-	-
Dicoma anomala Sond.	SV: Bloemfontein	15.0	-	-	-		
Pentanisia prunelloides (Klotzsch ex Eckl. & Zeyh.) Walp.	SV: Bloemfontein	-	7.0	1.0	1.0	-	-
Mean of positive samples ± standard deviation		15.0	9.6 ± 2.5	8.4 ± 7.7	1.0	4.0	81.3 ± 91.1

Only positive sample results have been shown; Concentrations in µg/kg; Not detected (-). SV-Street Vendor; MS-*Muthi* Shop.

3. Discussion

3.1. Aflatoxin (AFB_1)

Aflatoxins have been reported as mycotoxins of human importance [33]. There are various worldwide reports on aflatoxin contamination in medicinal plants. The current study findings seem to be consistent with a report by Aiko and Mehta, in their study of 63 Indian medicinal herbs samples only one sample tested positive for aflatoxin B [34]. In another study of African traditional herbal medicines sold in South Africa (Tshwane-Pretoria and Cape Town), all 16 samples were not contaminated with aflatoxins [17]. A study in Italy found that all samples of medicinal plants, aromatic herbs and herbal infusions were not contaminated with aflatoxins [29].

Another study of 500 herbal plants in Poland reported all samples to be safe from aflatoxins [35]. This is quite similar to the current study findings which only found one sample to be contaminated with AFB_1. In contrast, the authors of [36] reported aflatoxin contamination in 58.9% of herbal tea samples from Moroccan market. Tassaneeyakul et al., reported aflatoxin contamination in herbal medicinal plant products in Thailand, in the range of 1.7–0.0000143 µg/kg in 5 out of 28 samples, which is lower than the present findings [37]. The AFB_1 concentration reported in our study was above the results reported by Yang et al., with AFs (up to 32 µg/kg) in 3 of 19 samples of Chinese herbal medicines [38]. Commonly used Nigerian indigenous crude herbal preparations tested positive for aflatoxin contamination in the range of 0.004–0.345 µg/kg, which is also lower than the current study findings [18]. AFB_1 has also been reported in kava kava at a concentration of 0.0005 µg/kg. In the same study, other botanical roots' samples tested negative for aflatoxins [39] In China, the authors of [40] reported one sample of medicinal materials of radix and rhizome to be contaminated with AFB_1 (5 µg/kg).

The optimum conditions for aflatoxin production by *Aspergillus flavus* and *A. parasiticus* species are at (0.94–0.99 a_w) and temperatures (25–37 °C) [41,42]. The climate of the Free State province, especially the summer temperatures, might contribute to aflatoxin contamination in medicinal plants whilst another factor might be the climatic conditions where the plants are collected: Gauteng as well as the KwaZulu Natal major markets. The concentrations reported in this study are above the permissible limit for AFB_1 of 5 µg/kg and total AFs (10 µg/kg) [43]. The AFB_1 level reported in the study is also above maximum

limits of 2 μg/kg for AFB_1 and 4 μg/kg for total aflatoxins in herbal drugs set by the European Pharmacopoeia [44] as well as Liu et al. [45], who proposed maximum limits of 5 μg/kg and 10 μg/kg for AFB_1 and total aflatoxins, respectively. The presence of AFB_1 sheds light on the possibility of contamination of medicinal plants by aflatoxins. Therefore, consumers of medicinal plants sold in the markets might be at risk of mycotoxicosis due to aflatoxin contamination.

3.2. Deoxynivalenol (DON) and Nivalenol (NIV)

DON and NIV were not detected in all the samples that were analyzed in the current study. This outcome contrasts with a study conducted in Spain which found 62% of the 84 types of aromatics and/or medicinal herb samples analyzed were contaminated with DON [46]. A study of Chinese medicinal herbs and related products reported DON (17.2–50.5 μg/kg) contamination in 3 out of 58 samples [47]. A most recent study by Darra et al. [48] in Lebanon on multi-mycotoxin occurrence in commercial spices and herbs found DON (12% in spices, 3% in herbs) but NIV was not detected.

In Latvia, DON was detected in 45% of marketed herbal tea samples at concentrations of 129 μg/kg in the herbal blend and 5.463 μg/kg in wormwood tea [49]. Whilst previous studies have detected the presence of DON and NIV, the absence of these mycotoxins may be attributed to the environmental conditions which do not favor the production of these mycotoxins by fungi species. The European Commission has set limits of DON at maximum of 200 μg/kg for processed cereal-based food and 1250 μg/kg for unprocessed cereals [50]. In South Africa, DON is regulated and for maize or barley ready for human consumption they may not contain more than 1000 ug/kg of deoxynivalenol [43]. However, regulatory limits have not yet been provided for NIV.

3.3. Fumonisins (FB_1, FB_2, FB_3)

In accordance with the present results, previous studies have demonstrated that medicinal plants can be contaminated with fumonisins. In Turkey, 2% of 115 medicinal plants and herbal tea samples tested positive for FB_1 at levels of 0.00016 and 0.001487 μg/kg [51]. The current study findings were lower than previous studies in South Africa [16], where it was reported in the Eastern Cape that only 4 out of 30 medicinal wild plants samples tested were contaminated with FB_1 (8–1553 μg/kg. In another study of African traditional herbal medicines sold in Tshwane and Cape Town, 81% of 16 samples were found contaminated with FB1 (14–139 μg/kg) [17]. These earlier studies concluded that FB_1 contamination was more common in South African medicinal herbs whilst the current study found FB_2 to be the predominant fumonisin derivative.

Samples of black tea and medicinal plants sold in Lisbon supermarkets in Portugal 65% tested positive for FB_1 (range, 20 to 700 μg/kg) whilst none were contaminated with FB_2 [52]. Han et al. [53] reported that more than 50% of 35 samples of traditional Chinese medicines tested positive for fumonisins' contamination (0.58–88.95 μg/kg). In a recent study of mycotoxin contamination in Menthae haplocalycis, Luo et al., reported FB_1 and FB_2 in the samples analyzed [54]. The presence of fumonisins' contamination has been reported before which demonstrates the risk to consumers, the need for continuous monitoring and in vitro studies in exposure risk assessments.

In South Africa, fumonisin is regulated for raw maize grain intended for further processing, that may not contain more than 4000 μg/kg of FB_1 and FB_2, whereas for maize flour, maize meal ready for human consumption has a limit of 200 μg/kg for FB_1 and FB_2 whole commodity [43] The present study findings for total fumonisins were below the regulatory limits, but this does not absolve the consumer from fumonisin-mycotoxin health risks.

3.4. Ochratoxin (OTA)

In Poland, 49% of the 79 samples of herbs analyzed for natural occurrence of OTA contamination tested positive whilst 22.3% exceeded OTA acceptable limits [55]. The

OTA concentration from this study was higher than the results of Roy and Kumar [56] who reported that 44% of 129 herbal samples destined for Ayurvedic medicines were contaminated with OTA (range 0.3–0.00234 µg/kg). Aziz et al., reported higher OTA levels compared to our findings; in their study, 3 out of 17 medicinal plant samples were contaminated by OTA at a mean concentration of (20–80 µg/kg [25]).

In addition, Bresch et al., reported OTA contamination in 50% of the 19 licorice samples (range, 0.3 to 216 µg/kg) [57]. A study on Chinese medicinal plants reported OTA presence in 44% of the 57 samples analyzed (range 1.2–158.7 µg/kg) [38]. In a recent study Ochratoxin A (OTA) was detected in 10% of herbal teas marketed in Latvia at concentrations that ranged between 2.99–30.3 µg/kg [49].

According to EFSA, OTA has been found in breast milk, which could represent a possible health concern for breast-fed infants [58]. Shim et al., reported an OTA transfer rate of (12.72–61.33%) from herbal medicines to decoctions indicating that the use of mycotoxin-contaminated medicinal plants presents a health risk to the consumers after consumption of such products [59]. OTA is not regulated in South Africa, however, according to the European Union Commission Regulation [50], the maximum residue level (MRL) for OTA in nutmeg, ginger, turmeric, black and white pepper, licorice root and its extract, the legislative limit varies from 15 µg/kg to 80 µg/kg. In the current study, the OTA concentration was below the set limit as well as the European regulatory standard (5 µg/kg in unprocessed cereals) [50].

3.5. Zearalenone (ZEN)

The mean concentration for zearalenone recorded in this study was lower than the one reported in China wherein all nine samples of the coix seed medicinal herb tested positive for ZEN (range, 18.7–211.4 µg/kg) [60]. Similarly, another study in China [61] also reported ZEN contamination of coix seeds (68.9 to 119.6 µg/kg). ZEN was not detected in an earlier study of 84 medicinal plant samples using direct determination methods [26]. Different countries have set a maximum limit for ZEN ranging from 20 to 1000 µg/kg in raw and processed food items [50]. In our study, ZEN contamination levels (7–183 µg/kg) did not exceed the permissible limits. ZEN-advanced pubertal changes in young children have been reported in Puerto Rico and gynecomastia with testicular atrophy has been reported in rural males in Southern Africa [62,63].

3.6. Multiple Mycotoxin Contamination

In this study, the mycotoxin occurrence was mainly extracted from the fumonisin derivatives (FB_1, FB_2, FB_3). Mycotoxin co-occurrence has been reported in previous studies of medicinal plants. In Spain, all 84 samples of medicinal and aromatic herbs analyzed showed multi-contamination with AFs, OTA, ZEN, FBs, DON, T-2 toxin and citrinin [46]. Another study reported contamination in 20.58% of the powdered herbal samples with mycotoxins (total aflatoxins, sterigmatocystin, citrinin) [64]. In an analysis of ginger products, aflatoxins and OTA were detected in 67% and 74% samples, respectively, with a range of 0.001–0.03 ng/kg [65]. A study by Koul and Sumbali, [66], found the presence of ZEN and DON in 13.07% and 6.92% of 130 samples of medicinally important dried rhizomes and root tubers. Veprikova et al., analyzed herbal-based dietary supplements for the presence of 57 mycotoxins. The study reported Fusarium trichothecenes, ZEN and ENs and Alternaria as the main mycotoxins and mycotoxin co-occurrence of ENs, HT-2, T-2 and Alternaria toxins [67].

A simultaneous analysis of multiple mycotoxins in 44 samples of *Alpinia oxyphylla* by UPLC-MS/MS detected AFB_1, ZEN, OTA, FB_1 and FB_2 in four moldy samples [68]. Another study of multiclass mycotoxins in Chinese medicinal and edible lotus seeds found three of the ten batches of samples tested positive for AFB_1, FB_2, T-2 and ZEN [69]. An investigation into the presence of multi-class mycotoxins in 40 batches of *Menthae haplocalycis* samples found the most common mycotoxin was tentoxin, followed by alternariol, alternariol monomethyl ether, ZEN, FB_2, FB_3, OTA, AFB_1, AFB_2, AFG_1 and T-2 toxin [54].

Reinholds et al. [49] analyzed 60 samples of herbal teas from Latvia drugstores for the presence of 12 mycotoxins. Among the dry tea samples, 90% were positive for at least one–eight mycotoxins. A study on teas and medicinal plants used to prepare infusions in Portugal reported that 84% of the analyzed samples tested positive for at least one of the mycotoxins [70]. Narvaez, in the analysis of the presence of 16 mycotoxins in botanical nutraceuticals, reported a co-occurrence in 4 out of 10 samples (EN B1, EN A and EN A1). Meanwhile, the prevalent mycotoxins were ZEN (60%) and EN B1 (30%) in samples analyzed [71]. A recent study by Caldeirão et al. [72], analyzed 58 herbs from Brazil for the presence of 14 mycotoxins by LC-MS/MS. Mycotoxin multiple contamination (range 1–8) was reported in 72% of the samples. The most prevalent mycotoxins were enniatins (EN), beauvericin (BEA), sterigmatocystin (STE) and HT-2 toxin, whilst FB_1, FB_2, and T-2 were not detected in any of the samples. Furthermore, the concentration of mycotoxins in the herbal infusions was 88% lower than in the raw herbs.

The percentage of positive samples and mycotoxin co-occurrence of mycotoxins varied among the different studies. This can be attributed to the sensitivity of the methods used and the wide range of mycotoxins analyzed as compared to the current study which only investigated the presence of eight mycotoxins. The reports from preceding studies further indicate that multiple mycotoxin contamination in medicinal plants, herbs and herbal products is cause of concern. Therefore, there is need for a comprehensive analysis of other emerging mycotoxins in routine monitoring of medicinal plants and their products.

The presence of mycotoxins in human food and animal feed increases the risk of endemic diseases such as malaria, hepatitis and HIV with consequent acute and chronic effects [8]. The lack of epidemiological studies, focusing on co-exposure to multi-class mycotoxins and associated health outcomes, is partly attributable to the absence of valid biomarkers [8,73]. A study of 53 South African women, found eight single or combined mycotoxins in urine samples including: DON; FB_1; OTA; and ZEN [73]. In another study conducted in Cameroon, the authors reported the detection of 11 single or combined mycotoxins and their metabolites in 63% of 175 urine samples including AFM_1, OTA and DON [74]. The presence of more than one mycotoxin demonstrates the possibility of mycotoxin exposures from single or multiple sources not limited to food but also from other non-food sources as reported earlier. Therefore, the contribution of medicinal plants as source of mycotoxin exposures should not be underestimated. Whilst co-occurrence has been reported in medicinal plants by several previous studies, present study findings warrant further research to analyze for a wide range of mycotoxins, especially the ones not frequently studied/reported.

4. Conclusions

The study evaluated the presence of mycotoxins (AFB_1, DON, FBs, NIV, OTA, and ZEN) as they have been reported to be the major mycotoxins of public health importance. The findings indicate the prevalence of mycotoxin contamination in frequently traded medicinal plants in South Africa. Mycotoxins pose a health risk to consumers due to the additive or synergistic effects of mycotoxins. Taking into consideration the frequency of use, dietary intakes and individual susceptibility among other factors, consumers are at an increased risk from mycotoxins and their adverse health effects. The current study's findings, supported by previous urinary biomarkers' assessment reports, demonstrate that consumers of medicinal plants are at risk despite the low concentration levels recorded for some mycotoxins in medicinal plants and their products. This is the first study in the Free State Province, South Africa to investigate multiple mycotoxin contamination in marketed medicinal plants. There is therefore a need for routine monitoring of multiple mycotoxins and regulations as well as human exposure assessments using biomarker analysis. Inspections of storage and trading conditions, including regulation of trade, are required to ensure that the trade in medicinal plants is conducted in environments that do not favor the growth of fungi and mycotoxin production.

5. Materials and Methods

5.1. Standards and Reagents

The mycotoxin standards comprising of Aflatoxin B_1 (AFB_1), Zearalenone (ZEN), Nivalenol (NIV), Deoxynivalenol (DON) and Ochratoxin A (OTA), were obtained from Sigma-Aldrich (Bornem, Belgium). Fumonisin (B_1, B_2, B_3) was purchased from Promec Unit (Tynberg, South Africa). Acetonitrile (VWR International, Zaventem, Belgium) and methanol (Biosolve, Valkenswaard, The Netherlands), Formic acid (≥98%) (Merck, Darmstadt, Germany). All reagents were of analytical grade.

5.2. Sample Collection and Preparation

A survey was carried out throughout the Free State province, South Africa where 48 vendors were asked to list their top ten selling medicinal plants. Participants listed 165 medicinal plants. The plants which had a Frequency Index (percentage frequency of mention for a single species by informants) ≥ 10 were selected for further analysis. A total of 34 samples from 32 plant species (Table S1) were randomly selected and purchased from *muthi* shops and street vendors. The samples that were procured from the *muthi* shops (16) and street vendors (18) comprised of roots, bark, leaves, stems and bulbs. Samples were collected in a dry state in sterile zip-lock plastic bags and immediately transported to the CAFSaB laboratory at the Central University of Technology. Samples were further dried to reduce moisture content in a laminar air flow dryer (Lasec). All dried samples were milled using a Kinematica Polymix PX-MFC90D (Kinematica AG, Luzern, Switzerland) to less than 0.5 mm particle size. Homogenized samples of 30 g were divided into two, for mycotoxin and microbial analysis. Samples were stored in sterile zip-lock bags at 4 °C to inhibit mycotoxin production and fungal growth until analysis.

5.3. Mycotoxin Extraction

A simple solvent extraction method with no sample clean-up was used as described by Spanjer [27]. Homogenized samples were accurately weighed (approx. 5 g) using an analytical balance (3 dp) into a 50 mL tube. Extraction solvent of 20 mL of water/methanol/acetonitrile (2/1/1, *v*/*v*) was added and sonicated for 60 min. Then, 1 mL of sample was aliquoted into a 2 mL Eppendorf tube and double diluted with 75% water; 25% methanol solvent and centrifuged for 5 min at 13,000 rpm. A total of 1 mL of the diluted sample was aliquoted into an analysis vial for analysis.

5.4. Equipment Calibration

A calibration graph was created by plotting the obtained peak area or peak height for each standard working solution against the mass of each mycotoxin injected. Each mycotoxin peak in the chromatogram was identified by comparing the retention times with those of corresponding reference standards. The quantity of mycotoxins in injected eluate was determined by comparison to the respective standard curves of each mycotoxin standard.

5.5. Liquid Chromatography Tandem Mass Spectrometry

A Waters Acquity Ultra Performance Liquid Chromatography (UPLC) apparatus coupled to a Xevo Triple Quadrupole Tandem Mass Spectrometer (TQMS) (Waters, Milford, MA, USA) was used for high resolution UPLC/MS/MS for the detection and quantification of mycotoxins. A symmetry Waters column UPLC BEH -C18 (100 mm × 2.1 id; 1.7 µm particle size) attached to a guard column (10 mm × 2.1 mm i.d.) (Waters, Zellik, Belgium) was used. A fixed sample injection volume of 2 µL was used. Mobile phase solvent A consisted of acidified water with 0.1% formic acid (10/1, *v*/*v*) and mobile phase solvent B of 0.1% formic acid in acetonitrile acidified with 0.1% formic acid (10/1, *v*/*v*). Multiple mycotoxins were separated in the mass spectrometer operated using selected multiple reaction monitoring channels (MRM) in positive electrospray ionization mode (ESI+). ESI conditions were optimized as follows; capillary voltage, 3.5 V; cone voltage range, 15–50 V; collision energy range, 10–40 eV; source temperature, 140 °C. Nitrogen was used as the

desolvation gas, desolvation temperature of 400 °C; desolvation gas, 800 L/h and cone gas, 50 L/h.

The gradient elution program (illustrated in Figure S1) at initial conditions of 98% A (Water + 0.1% formic acid), held for 0–0.5 min at a flow rate of 0.35 mL/min was used. This was followed by a slow gradient change of solvent A to 60% from 0.5–7 min. From 7–10 min there was another gradient change of solvent A to 30%. A rapid gradient change ensued for solvent A to 5% from 10–11 min. There was another gradient change of solvent A to 0% from 11–12 min. This was followed by a quick gradient change to initial conditions of 98% solvent A from 12–12.1 min. After that, an isocratic period of 98% of solvent A was kept for 12.1–14 min. The column was reconditioned with solvent B (Acetonitrile + 0.1% formic acid) for 5 min before the next injection. The total analytical run time was 14 min through a linear decrease of mobile phase.

5.6. Data Acquisition and Analysis

The frequency index (FI) used to select the plants used in this study was calculated using the formula FI = (FC ÷ N) × 100. FC is the number of informants who mentioned the use of the species, and N is the total number of informants. N = 48 in this study. Plant names were documented in the local languages, mostly Sotho and Zulu, and scientific names identified from literature.

MassLynx and QuanLynx software's version 4.1 (Micromass, Manchester, UK) were used for data acquisition and processing. Descriptive statistics (mean, range, maximum and the frequency of the data obtained in this study) were calculated using Microsoft Office Excel 2016.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/toxins14100690/s1, Figure S1: Gradient elution program; Table S1: Medicinal plants screened for mycotoxins.

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Institutional Review Board Statement: The study protocol was approved by the University Research and Innovation Committee (URIC) of the Central University of Technology, Free State. Ethical clearance was obtained from the University of Free State's Environment and Biosafety Ethics Committee (UFS-ESD2019/01831504) and the Health Sciences Research Ethics Committee (UFS-HSD2019/1226/2605) on 15 April 2020 and 4 May 2020 respectively.

Informed Consent Statement: Participation in the study was voluntary, informed consent was obtained from all participants involved in the study.

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