ASSESSMENT OF MYCOTOXIN CONTAMINATION IN TRADITIONAL LEAFY VEGETABLES SOLD IN THE FREE STATE, SOUTH AFRICA.

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Declaration of independent work

DECLARATION WITH REGARD TO INDEPENDENT WORK

I, **LIAKO MOHALE,** student number __________, do hereby declare that this research project submitted to the Central University of Technology, Free State for the Master of Engineering in Mechanical Engineering, 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.

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SIGNATURE OF STUDENT **DATE**

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Summary

Traditional leafy vegetables (TLVs) are an essential supplement to grain-based diets. They are a valuable source of nutrition in rural areas where exotic species are unavailable and contribute substantially to protein, mineral and vitamin intake. However, they are seasonal and highly perishable, making them susceptible to contamination. This study conducted a market survey for TLVs in the Free State Province, South Africa, to determine the most consumed TLVs, preservation methods, packaging and storage conditions. Fungal contaminants and associated mycotoxins were identified. A descriptive cross-sectional survey design was used to collect data from formal and informal markets in the Free State's rural and urban areas. Interviews were conducted with 60 vendors that sold TLVs used either as food or medicines using semi-structured questionnaires. Data collected from the interviews were analysed using descriptive statistics. The interviews mentioned ten traditional leafy vegetables, but six species, *Urtica dioica* L.*,* *Rorripa nudiuscula* Thell., *Lepidium capense* Thunb.*, Amaranthus hybridus* L.*, Chenopodium album* L. and *Cucurbita maxima,* were readily available and collected for mycological contamination tests. Nine fungal species; *Epicoccum sorghinum, Alternaria alternate, Phoma sp., Cladosporium sp., Rhizopus oryzae, Nothophoma quercina, Fusarium sp*., *Didymella glomerata* and *Didymella macrostoma,* were present on the vegetable samples evaluated. To further assess the safety of TLVs, mycotoxin analysis and quantification were carried out using a rapid and sensitive ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-ESI-MS/MS). The samples were tested for Aflatoxin B1, Deoxynivalenol, Nivalenol, Ochratoxin A, Zearalenol, Fumonisin B1, Fumonisin B2, and Fumonisin B3. Of the tested vegetable samples, only *Amaranthus hybridus* L. and *Chenopodium album* L. had traces of the mycotoxins; Fumonisin B1 and Fumonisin B2.

The results obtained in this study showed that TLVs are safe for consumption. Nevertheless, as much as the mycotoxins detected were below set limits, cumulative exposure may pose a health risk to consumers. Consequently, TLVs traders and consumers must be made aware of the risks associated with mycotoxins and educated on the importance of proper hygiene, preservation and storage practices

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CHAPTER 1

INTRODUCTION

1.1 General background

Africa has a wide variety of traditional leafy vegetables with high micronutrient levels, which can help alleviate malnutrition and ensure food security among African populations (Oguntoyinbo *et al*., 2016). Traditional leafy vegetables (TLVs) are plants with leaves that are traditionally used as vegetables by both rural and urban populations (Shayanowako *et al*., 2021). For instance, South Africa with a significant cultural and biological diversity, have many people still depending on indigenous and naturalised plants for their daily food, shelter, fuel, medicine and other necessities of life (Otang-Mbeng and Mashabela, 2020; Odhav *et al*., 2007). More than 100 plant species have been identified for use as traditional leafy vegetables in South Africa (Dweba & Mearns, 2011), including *Bidens pilosa* L., *Amaranthus hybridus* L., *Cleome gynandra* L., *Chenopodium album* L., *Solanum retroflexum*, and *Cucurbita maxima* (Taleni, Nyoni, and Goduka, 2012).

Traditional leafy vegetables are considered an essential supplement to grain-based staple diets. These vegetables are referred to as moroho in Sotho, imfino in Zulu and Xhosa, and morogo in Tswana and Pedi (Otang-Mbeng & Mashabela, 2020; van Rensburg *et al*., 2007; van der Walt *et al*., 2006). In preparation for a relish, a single plant species may be used, or different species may be combined. Additional ingredients to the dish include salt, butter, milk, tomatoes or onions, based on the individual's preference (Mokganya, Mushaphi and Tshisikhawe, 2019; Uusika *et al*., 2010). People consume traditional leafy vegetables for different reasons, including their taste, nutritional value, cost, and accessibility (Punchay *et al*., 2020; Matenge *et al*., 2011).

According to van Rensburg *et al*. (2007), the consumption of TLVs differs among households, depending on the social status, the season of the year and the degree of urbanisation. Traditional leafy vegetables are utilised mainly by poor households, especially those living in rural areas (Mncwango *et al*., 2020; van Rensburg *et al*., 2007). Communities in rural areas have easy access to TLVs, unlike those in urban areas. They can harvest vegetables from the wild, farms and home gardens, whereas urban dwellers experience limited or no access to such locations (Uusiku *et al*., 2010).

A decline in the consumption of traditional leafy vegetables has been noted over the years as a result of the introduction of exotic vegetable, urbanisation and modernisation, added to the fear of stigmatisation because they are considered poor people’s food by those of a higher class (Uusiku *et al*., 2010). Other factors that can affect the consumption of TLVs are gender, age, cultural background and geographical location (van Rensburg *et al*., 2007). Despite the decline in consumption of traditional leafy vegetables, some people still show interest in it and are willing to utilise them if only they can receive more information about them and how they are prepared (Gido *et al*., 2017).

After harvesting, TLVs are prepared and stored using traditional methods, and the surplus is sometimes dried to be used during off seasons (Uusika *et al*., 2010). The most common processing methods used by small-scale farmers include fermentation, sun drying, blanching and solar drying (Mepba, Eboh, and Banigo, 2007). It is during these processes that fungi can colonise the vegetables and may accumulate compounds called mycotoxins. Mycotoxins are fungal secondary metabolites produced by toxigenic strains of fungi that contaminate various food substances and crops (Awuchi *et al*., 2021). This normally occurs in the field, while handling, during transportation and storage of the vegetables (Patriarca and Pinto, 2017).

Processing of TLVs may also result in multi-mycotoxin contamination. Examples of mycotoxigenic genera include Aspergillus, Penicillium, Fusarium, and Alternaria. Mycotoxins cause different toxic effects, as they are carcinogenic, neurotoxic, teratogenic, and immunotoxic (Sun *et al*., 2022). In addition, people living with a weakened immune system are more predisposed to illness and infection than healthy individuals. The most problematic mycotoxins from a human and domestic animal health perspective are aflatoxins (AFs), ochratoxins (OTs), fumonisins (FUMs), zearalenone (ZEA), trichothecenes (TH) and deoxynivalenol (DON) (Darwish et al., 2014). People may be exposed to these toxins through inhalation, ingestion or contact with contaminated foodstuffs (Awuchi *et al*., 2021).

The occurrence of mycotoxins in food renders them unsafe for consumption (El-Sayed *et al*., 2022). According to Awuchi and colleagues (2021), mycotoxins are emerging global contaminants that affect both human and animal health. Therefore, it is vital to utilise various measures necessary to reduce the risk of contamination. These measures include modifying agricultural practices, employing better preservation methods and storing vegetables under conditions that will evade the multiplication process involving mycotoxins (Nada *et al*., 2022). Despite the attempts to eliminate the presence of mycotoxins, they are still persistent and identified as an unavoidable risk. Consequently, there is a dire need to develop and implement working systems to minimise the risks of mycotoxins in foods (Barkai-Golan and Paster, 2011).

1.2 Problem statement

Mycotoxin contamination of foods and feeds remains a multifaceted challenge to food safety, public health and the economy (Imade *et al*., 2021). Exposure to dietary mycotoxins is more likely to occur in parts of the world where food handling methods and storage practices are inadequate, and where there are few regulations to protect consumers (Klich *et al*., 2003). The presence of mycotoxins in food is often overlooked in Africa due to public ignorance about their existence. Mycotoxins like aflatoxins and fumonisins are widely spread and are mainly reported to be a major problem in staple foods in Africa (Wagacha & Muthomi, 2008).

Traditional leafy vegetables are produced organically or collected from the wild, consequently, any fungus that could be present in them is not managed or controlled by pesticides/fungicides, which places them at a higher risk of fungal contamination that could eventually cause mycotoxin diseases upon consumption (Mutie *et al*., 2020; Machado-Moreira *et al*., 2019). One important issue with the safety of plants, such as traditional vegetables and medicinal herbs, is the control of mycotoxins (Altyn and Twaruzek, 2020) and there is a dearth of information and research on the prevalence and concentrations of mycotoxins in traditional leafy vegetables in South Africa. Therefore, it is necessary to investigate the occurrence of mycotoxigenic fungi and their associated mycotoxins, that could potentially contaminate traditional leafy vegetables.

1.3 Aim of the study

This study aimed to assess mycotoxigenic fungi and multi-mycotoxin contamination in traditional leafy vegetables in Free State, South Africa.

1.3.1 Specific objectives

To achieve the aim of the study, the following objectives were considered:

i. To document the traditional leafy vegetables sold in markets and/or consumed in the Free State province, South Africa.
ii. To determine the preservation methods, packaging and storage conditions for traditional leafy vegetables sold in the Free State province markets.
iii. To isolate and morphologically identify fungi found in traditional leafy vegetables sold in Free State, South Africa.
iv. To analyse the mycotoxins, present in traditional leafy vegetables in the Free State province, South Africa.

1.3.2 Study workflow

The flow chart below represents steps to put in place to achieve the aim of the study. The literature review was done using search engines such as Google Scholar, Science Direct, and Researchgate. The survey of traditional leafy vegetables markets in Free Sate was carried out using questionnaires to capture data. After that, the identified vegetables from the survey were collected using sterile polyethene bags. Upon arrival at the laboratory, the samples were prepared for isolation and identification of fungi using serial dilution and polymerase chain reaction (PCR) methods. Lastly, traditional leafy vegetables samples were tested for mycotoxins using ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-ESI-MS/MS).

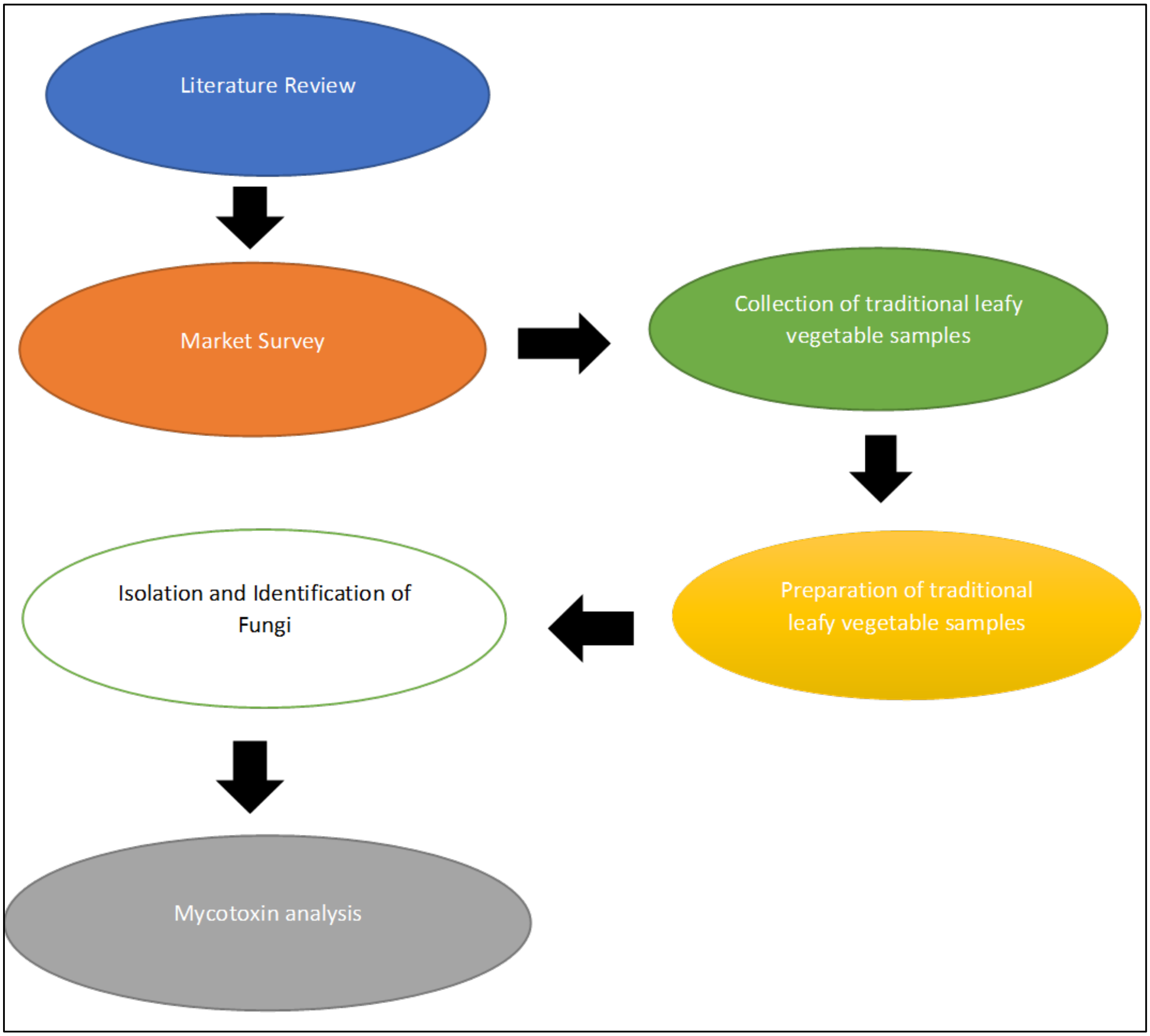

Figure 1.1: A flow chart showing different steps followed in carrying out the study

1.4 Chapter overview

The dissertation consists of six chapters, clearly delineated to give a comprehensive understanding of the study and guided by the title.

Chapter 1 provides background information on the purpose of the study, the importance of TLVs, and their probable contamination with mycotoxigenic fungi and Chapter 2 comprises the literature review that gives detailed insight into current knowledge pertaining to TLVs including the descriptions of nutritional values, production and utilisation of TLVs; post-harvest handling, preservation, storage and trade; sources of contamination of TLVs targeting the mycotoxigenic fungi and the produced mycotoxins. Lastly, the effects of mycotoxins on health are outlined.

The survey of traditional leafy vegetables sold in the Free State province forms Chapter 3. It involves different aspects of TLVs; the vegetable species available in the markets and preservation and storage practices in the said province. The fourth chapter reports on the molecular identification of fungi in TLVs using the PCR based method. In contrast, the fifth chapter covers the identification of mycotoxins produced by fungi recovered from TLVs using UPLC-ESI-MS/MS. Finally, the last chapter constitutes the general discussion of the results obtained in the study, conclusions and recommendations.

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CHAPTER 2
LITERATURE REVIEW

2.1 Background and description of traditional leafy vegetables

Traditional leafy vegetables (also known as African leafy vegetables) are plants with leaves or aerial parts being integrated into a community's culture for use as food over a large span of time (Towns & Shackleton, 2019; Chweya & Eyzaguire, 1999). They grow in the wild or are cultivated in home gardens. These vegetables are filled with active compounds such as carotenoids and polyphenols, making them food-medicine (Guarrera and Savo, 2013). Consequently, traditional leafy vegetables are considered important food components for humans in rural, peri-urban, and some urban settings in sub-Saharan Africa (Weinberger & Msuya, 2004). The use of leafy vegetables in South Africa has been an age-long practice; the Khoisan people, who lived in Southern Africa for at least the past 120 000 years, survived by gathering these plants from the wild (van Rensburg *et al*., 2007).

Traditional leafy vegetables are also referred to as indigenous leafy vegetables (ILVs), traditional African leafy vegetables (TALVs), indigenous African vegetables (IAVs), African indigenous leafy vegetables (AILVs), African leafy vegetables (ALVs), and wild vegetables (Gido *et al*., 2017; Mavengahama *et al*., 2013; van Rensburg *et al*., 2007). They are highly recommended because they have demonstrated a relatively high nutritional value compared to the introduced varieties (Uusiku *et al*., 2010.). Their consumption gives diversity to the daily food intake, adding flavour and appetite to the diet (Kumar, Kumar and Shekhar, 2020).

2.2 Examples and ethnobotanical description of common TLVs in South Africa

Plants which are utilised as traditional leafy vegetables include cowpea leaves (*Vigna unguiculata* L. Walp.), cat whiskers (*Cleome gynandra* L. and *Cleome monophylla*), cucurbits (*Cucumis* sp, *Citrillus* sp, and *Cucurbita* sp), and amaranth (*Amaranthus hybridus* L., *Amaranthus thunbergii*) (Vorster *et al*., 2007). Amaranths are herbaceous annual growing plants whose leaves are long-stalked with whitish veins, and the flowers vary significantly according to the species. *Amaranthus hybridus* and *Amaranthus thunbergii* are the two most prevalent species, widely employed as green leafy vegetables in traditional African settings. In addition to their value as food plants, amaranths have also been known to have medicinal applications.

The genus *Cleome*, commonly known as cat's whiskers or African cabbage, is an erect, herbaceous annual herb growing to a height of 1.5m and being influenced by environmental conditions (Van Wyk and Gericke, 2003). Examples include *Cleome gynandra*, a wild-growing semi-cultivated tropical leafy vegetable in many parts of sub-Saharan Africa, particularly in Eastern and Southern Africa (Moyo and Aremu, 2022). The tender leaves and flowers of the African cabbage are boiled and eaten while served as a potherb, a tasty relish, stew, or a side dish throughout Africa (Takaidza, 2023). The leaves are bitter; therefore, they are cooked with other vegetables, including cowpea (*Vigna* spp), amaranth (*Amaranthus* sp) or black nightshade (*Solanum nigrum* L.) (Managa and Nemadodzi, 2023).

Cowpea (*Vigna unguiculata L. Walp*.) is one of the most diverse and nutritious grain legumes cultivated in sub-Saharan Africa, South America, Asia, parts of Southern Europe and the south-eastern and south-western regions of North America (Ehlers & Hall, 1997). It is a mainly cultivated leafy vegetable (Adekalu & Okunade, 2006). Cowpeas are good sources of antioxidants, flavonoids, phenols, carotenoids, calcium, and vitamin C (Chaurasia, 2020). Cowpea leaves are essential as a relish and can be prepared with oil, onions, tomatoes and coconut milk (Mduma, 2010). It is uncommon to preserve cowpea leaves as most consumers prefer them fresh.

The genus *Cucurbitaceae*, also called cucurbits, is a plant family of 118 genera and 825 species (Lira, Villasenor and Ortiz, 2002). The members are widespread throughout the tropics and temperate areas in the world (Singh, 1990). A few species of these plants were domesticated by humans and are now a part of the staple crops worldwide (Lira, Villasenor and Ortiz, 2002). The most important ones to humans include cucumber, melon, watermelon, calabash, squash, and pumpkin (Baloglu, 2018). Many plants in this family are essential for different societies' cultures and economies (Lira, Villasenor and Ortiz, 2002).

Table 2.1: Some commonly available and consumed traditional leafy vegetables in southern Africa

Scientific names	Local and English names	Preservation methods	References
Amaranthus hybridus L. subsp. *hybridus* var. *hybridus*	Thepe (Tswana), Smooth pigweed	Drying in the shade	Department of Agriculture, Forestry and Fisheries, 2010
Amaranthus viridis L.	Theepe (Sotho), Slender amaranth	Drying in the shade	Department of Agriculture, Forestry and Fisheries, 2010
Amaranthus thunbergii Moq.	Thepe (Tswana), Thunberg's pigweed	Drying in the shade	Department of Agriculture, Forestry and Fisheries, 2010
Cleome gynandra L.	Lerotho (Tswana), Cat's whiskers	Sun-drying, Blanching and freezing	Department of Agriculture, Forestry and Fisheries, 2014
Cucurbita sp.	Morogo wa lephutse (Tswana), Squash / pumpkin leaves	Sun-drying	Chamba et al., 2017
Lepidium capense Thunb.	Qhela (Sotho), Peppercress	Sun-drying	Department of Agriculture, Forestry and Fisheries, 2014
Malva parviflora L. var. *parviflora* *Malva verticillata* L. var. *verticillate*	Tikamotse (Sotho), Cheeseweed mallow	Sun-drying	Messaoudi et al., 2015
Chenopodium album L.	Seruwe (Sotho), Goosefoot	Sun-drying	Singh, 1990
Urtica dioica L. *Urtica lobulata* Blume. *Urtica urens* L.	Bobatsi (Sotho), Stinging nettle	Blanching; Drying Freezing	Stewart, 2015
Lactuca tysonii (E. Phillips) C. Jeffrey *Sonchus dregeanus* DC.	Leharaswana (Sotho), Hare lettuce	Drying	Department of Agriculture, Forestry and Fisheries, 2010

2.3 General nutritional components of traditional leafy vegetables

Traditional leafy vegetables are sources of high-quality nutrition, including a great source of vitamins (A, C, B6 and B12), minerals (zinc, iron, and potassium), fibre, essential amino acids, and antioxidants necessary for a healthy diet and the prevention of diseases (Van der Hoeven *et al*., 2013.). Concisely, many traditional leafy vegetables have been reported to have health-protecting properties and uses. For example, *Amaranthus hybridus* L. is reportedly endowed with beta-carotene, calcium, iron, and vitamin C (Kwenin *et al*., 2011). A Zimbabwe study described amaranth's nutritional content to provide adequate nutrients even after undergoing different processing and preservation methods (Makobo *et al*., 2010).

Table 2.2: Nutritional components of some traditional leafy vegetables in the world

Traditional leafy vegetables	**Nutritional components**
Amaranthus hybridus L. subsp. *hybridus* var*. hybridus*	Protein, fibre, carbohydrates, calcium, magnesium, vitamin C, iron
Bidens pilosa L.	Protein, fibre, copper, magnesium
Cleome gynandra L.	Vitamin C
Chenopodium album L.	Protein, Vitamin C, iron, zinc, magnesium
Urtica dioica L.	Protein, fibre, carbohydrates, amino acids, flavonoids, potassium, zinc
Malva parviflora L. var. *Parviflora*	Iron, copper, zinc, magnesium
Cucurbita sp.	Fibre, vitamin A, vitamin C, vitamin E, manganese, magnesium, potassium
Lepidium capense Thunb.	Calcium, magnesium, potassium, dietary fibre, protein
Sonchus dregeanus DC.	Protein, fibre, fat, vitamin C, Beta-carotene
Rorippa nudiuscula Thell.	Vitamin C, protein, lipid, Beta-carotene

(Bvenura & Afolayan, 2015)

Traditional leafy vegetables are anticipated to contribute significantly to the global initiative of WHO to increase the consumption of fruits and vegetables in African countries

(Gido *et al*., 2017). However, as nutritious as TLVs are, they may harbor secondary metabolites called mycotoxins that may cause adverse health effects to animals and humans. Contamination of these vegetables with mycotoxins may occur in the field, during harvesting, processing, storage and marketing (Barkai-Golan and Paster, 2011). Conditions that make fungal contamination possible include temperature, humidity and tissue susceptibility (Afsah-Hejri *et al*., 2013).

2.4 Production and utilisation of traditional leafy vegetables

Even though the utilisation of TLVs is part of Africa's cultural heritage since they play a vital role in the customs, traditions, and food culture of African households (Mensah *et al*., 2008), their cultivation has declined drastically due to the excessive cultivation of field crops, during which the vegetables are chemically eliminated as they are considered as weeds (Lewu and Mavengahama, 2010). According to Mavengahama (2013), TLVs have been administered as food over a significant period. Still, they have not been widely domesticated and are not cultivated on a large scale, especially in South Africa. The occurrence and extent of cultivation of leafy vegetables in South Africa have been presented in Table 2.3.

Table 2.3: Traditional leafy vegetables commonly harvested from the wild or obtained through cultivation in South Africa

Traditional leafy vegetable	Harvested from the wild	Cultivated	Growth season
Abelmoschus esculentus Moench.	✓	✓	Summer
Amaranthus sp.	✓	✓	Summer
Bidens pilosa L.	✓		Summer
Brassica rapa L. subsp. *Chinensis*		✓	Winter
Chenopodium album L.	✓		Summer
Citrillus lanatus	✓	✓	Summer
Cleome gynandra L.	✓		Summer
Corchorus olitorius L.	✓		Summer
Cucumis melo L.		✓	Summer
Cucurbita sp.		✓	Summer
Galinsoga parviflora Cav.	✓		Summer
Momordica balsamina L.	✓		Summer

Portulaca oleracea L.	✓		Summer
Solanum retroflexum Dun.		✓	Winter
Vigna unguiculata (L.) Walp. subsp. *dekindtiana* (Harms) Verdc.		✓	Summer

(Maseko et al., 2017)Mycotoxin accumulation in vegetables during the production phase is influenced by several factors, including environmental conditions, fungal strain, and substrate (Daou *et al*., 2021). Weather contributes to the occurrence and development of crop diseases (Launay *et al*., 2014): high temperatures and humidity increase the risk of fungal growth and mycotoxin production (Daou *et al*., 2021). Rainy harvesting seasons are related to a higher content of mycotoxins in crops compared to drier harvesting seasons (Hines and Lorenzoni, 2023). However, the effect of harvest date on mycotoxin accumulation is not clear. Therefore, it is important to take into account the environmental conditions during the harvesting season when assessing the risk of mycotoxin contamination in vegetables.

2.5 Post-harvest handling and storage traditional leafy vegetables

Post-harvest handling describes the subsequent processes conducted immediately after removing a plant or a plant part from its growth media until the product reaches the final consumer in the desired form, including the packaging, quantity, quality, and price (Bremner, 2012). Post-harvest handling of vegetables is a critical step in ensuring the safety and quality of the produce. Fungal contamination of agricultural products, including vegetables, usually occurs during the post-harvest phase of the supply chain, which includes cooling, cleaning, sorting, storing and packaging (Food and Allergy Consulting and Testing Services (Pty) Ltd, 2020).

Post-harvest diseases are a significant cause of vegetable losses, with most diseases being caused by fungi such as *Alternaria, Aschochyta, Didymella, Phoma, Pythium, Rhizoctonia and Sclerotinia* (Tripathi, Tiwari and Behera, 2022). Vegetables should be stored in a cool, dry, and well-ventilated environment to reduce the risk of fungal growth and mycotoxin production (Nan, Xue and Bi, 2022). The ideal temperature for storing vegetables is between 0ºC to 15ºC, with a relative humidity of 85% (Giannakourou and Tsironi, 2021). High temperatures and humidity increase the risk of microbial contamination.

The risk of mycotoxin contamination can be mitigated through a combination of techniques to limit and prevent fungal contamination of foodstuffs and feed. According to Smith and Eyzaguirre (2007), there is a need to develop and promote appropriate handling and processing techniques to minimise post-harvest losses and ensure the regular supplies of these nutrient-rich vegetables from the time of harvest until they reach the consumers for utilisation. These techniques include temperature and humidity control, which reduces microbial growth (Food and Allergy Consulting and Testing Services (Pty) Ltd, 2020).

Mycotoxin contamination of vegetables can cause post-harvest losses (Nji *et al*., 2022). Therefore, it is essential to verify that products are safe by adhering to maximum limits of these toxins permitted in food and animal foodstuffs as determined by regulatory bodies and developing regulations for the informal food sector (Food and Allergy Consulting and Testing Services (Pty) Ltd, 2020).

2.6 Preservation of traditional leafy vegetables

Preservation methods are used to extend the shelf life of vegetables and fruits (Liu, Zhang and Bhandari, 2020). However, these methods have several disadvantages. Processes involved in vegetable preservation include blanching, a process conducted through heating in which the enzymes in the vegetables become inactivated either by dipping the vegetables in hot water or spraying them with steam. This is done before drying so that the dried vegetables can be refreshed more readily (Deng *et al*., 2019).

Drying involves the removal of water from food to extend its shelf-life without causing any significant changes to its qualities (Guiné, 2018). In a study by Faber *et al*. (2010), the vegetable leaves were dried in the sun or shade and reserved for consumption during winter. Sun drying is a traditional method of preserving vegetables that involves exposing them to the sun for several days until they are completely dry (Kiremire *et al*., 2010). While sun drying is an effective way to preserve vegetables, it has several disadvantages. Firstly, temperature cannot be controlled during the drying process, and food may get overheated (Arata and Sharma, 1991). This can lead to the loss of nutrients and vitamins in the vegetables (Sagar and Suresh, 2010). Secondly, sun drying is a slow process that can take several days, which increases the risk of contamination by insects, rodents and microorganisms (Alp and Bulantenkin, 2021).

2.6.1 Effects of Preservation on the traditional leafy vegetables

The preservation technologies impact the quality, microbiological safety and consumer acceptability of the TLVs, depending on the preservation method (Bulbula & Urga, 2018). The accumulation of mycotoxins in vegetables can occur when different types of preservation methods are employed (You *et al*., 2022). One common preservation method is drying, which can concentrate mycotoxins present in vegetables. When vegetables are dried, the moisture content is reduced, creating an environment where mycotoxins may become more concentrated (Misihairabgwi *et al*., 2019). This is especially true if the vegetables were already contaminated with mycotoxins before the drying process. (Nan, Xue, and Bi, 2022).

Another preservation method that can impact mycotoxin levels in vegetables is freezing. While freezing can slow down the growth of fungi responsible for mycotoxin production, it does not necessarily eliminate existing mycotoxins (Enikova, Stoynovska and Karcheva, 2020). Some mycotoxins are stable at low temperatures, and when vegetables are frozen without prior mycotoxin decontamination, these toxins may remain present and even become more concentrated as the water content in the vegetables freezes and forms ice crystals (Awuchi *et al*., 2021).

Lastly, the use of chemical preservatives or pesticides may also influence mycotoxin accumulation in vegetables (You *et al*., 2022). While these substances are intended to prevent the growth of fungi and reduce mycotoxin contamination, their effectiveness can vary depending on factors like application timing and concentration (Tudi *et al*., 2021). In some cases, improper or excessive use of chemical preservatives may lead to the development of mycotoxin-resistant fungi, ultimately allowing mycoxin levels ot persist or even increase over time (Olugbenga, Japhet and Victor, 2020).

2.7 Marketing of traditional leafy vegetables

The commercialisation of TLVs in South Africa is low and mainly focused on dried products (Mayekiso *et al*., 2021). Rarely are these vegetables found in supermarkets, but are traded primarily by street vendors and, in this manner, limiting their availability (Lyatuu *et al*., 2009). While TLVs offer a rich source of nutrients and cultural significance, the commercial production and distribution of these crops can be susceptible to fungal contamination (Alegbeleye *et al*., 2022). This issue arises from several factors, including the often decentralised and less regulated supply chains associated with TLVs (Victor *et al*., 2017).

Fungal contamination of TLVs can occur at multiple stages of the commecialisation process. From pre-harvest to post-harvest handling and storage, these vegetables are exposed to environmental conditions that may favor fungal growth (Afolabi *et al*., 2020). Inadequate sanitation practices, improper storage facilities, and a lack of awareness about fungal pathogens can contribute to contamination (Darwish *et al*., 2014). Moreover, the limited availability of effective fungicides or antifungal treatments specifically made for TLVs can exacerbate the problem.

The consequences of fungal contamination in commercially marketed TLVs multifaceted. Not only does it lead to economic losses for growers and distributors due to spoilage and reduced marketability, but it also poses health risks for consumers (Sanzani, Reverberi, and Geisen, 2016). Some fungal species produce mycotoxins, which are toxic compounds that can accumulate in contaminated vegetables and pose a threat to human health when consumed. (Nan, Xue and Bi, 2022).

2.8 Contamination of traditional leafy vegetables

Microbial contamination of TLVs is a significant concern in the food industry and public health. These vegetables, often grown in open fields and subjected to various environmental factors, are prone to contamination by a wide range of microorganisms (Alegbeleye *et al*., 2022). Bacteria, viruses, parasites and fungi can all find their way onto the vegetable leaves through contact with contaminated soil, water, animals or even human handling (Aworh, 2021). Such contamination poses serious health risks to consumers, as consuming these vegetables without proper washing or cooking can lead to foodborne illnesses (Balali *et al*., 2020).

The source of contamination in TLVs can vary: impoper hygiene practices during cultivation, harvesting, and processing can introduce harmful microorganisms (Alemu *et al*., 2019). Additionally, contaminated irrigation water, animal waste, or proximity to the source of pollution can contribute to microbial contamination. To address this issue of microbial contamination in TLVs, it is essential to implement food safety measures such as good agricultural practices (GAPs) and educating farmers, distributors and consumers about safe processing practices. (Machado-Moreira *et al*., 2019).

2.9 Mycotoxigenic fungi

Different fungi grow at different rates, determined by the temperature and water activity of the substrate they colonize (Ramakrishna *et al*., 1996). Based on their individual responses to these two factors, fungi can be differentiated into two groups, namely field and storage fungi. In the field, where moderate temperatures and water activities persist, fungi are almost always and constantly associated with exposed freshly decaying green parts of plants (Hudson, 1991). On the other hand, storage fungi colonize crops (grain and other plant materials) harvested, processed, and subsequently stored (Usha *et al*., 1993).

Mycotoxigenic fungi produce secondary metabolites known as mycotoxins, that grow on seeds, grains, and feed in the field or storage (Reddy *et al*., 2008). Among the numerous species of fungi, only about 100, belonging to the genera *Aspergillus*, *Penicillium*, and *Fusarium,* are known to produce mycotoxins. Table 2.2 shows examples of the mycotoxigenic fungi, the mycotoxins produced, commonly affected crops and their distribution worldwide. Of the 300-400 mycotoxins known, the most important include aflatoxins, ochratoxins, deoxynivalenol, zearalenone, fumonisin, and T-2 toxin (Karkashadze *et al*., 2022). Deoxynivalenol, zearalenone, T-2 toxin, and fumonisns are all mycotoxins produced by fungi of the genera *Fusarium* (Wagacha & Muthomi, 2008).

Table 2.4: Some examples of mycotoxigenic fungi, mycotoxins, geographical area and affected crops

Fungal species	Mycotoxins	Distribution	Crops
Aspergillus flavus *Aspergillus parasiticus* *Aspergillus bombycis* *Aspergillus pseudotamarii* *Aspergillus rambelli*	Aflatoxins (B1, B2, G1, G2, M1)	Europe Sub-Saharan Africa	Corn, cottonseed, peanuts, tree nuts
Fusarium graminearum *Fusarium culmorum*	Deoxynivalenol	Sub-Saharan Africa	Corn, wheat, oats, barley
Fusarium verticillioides *Fusarium proliferatum*	Fumonisns (FB1, FB2, FB3)	Asia Central America Sub-Saharan Africa	Corn, rice, sorghum, barley
Aspergillus ochraceus *Penicillium verrucosum* *Aspergillus niger* *Aspergillus carbonarius*	Ochratoxin A.	Southern-Northern America	Grains, raisins, barley, soy products
Fusarium graminearum	Zearalenone	Sub-Saharan Africa	Corn, wheat, barley, rye, sorghum

(Warth *et al*., 2012)

2.10 Mycotoxins

Mycotoxins occur naturally and frequently in food and feed (Dey *et al*., 2022). These mycotoxins include aflatoxins, ochratoxins, fumonisins, zearalenone, and deoxynivalenol (Dey *et al*., 2022). The natural occurrence of mycotoxins in vegetables has been demonstrated in many countries (Cinar and Onbaşı, 2019). However, reports on mycotoxin contamination of traditional African vegetables are exceptionally rare (Nafisa *et al*., 2017).

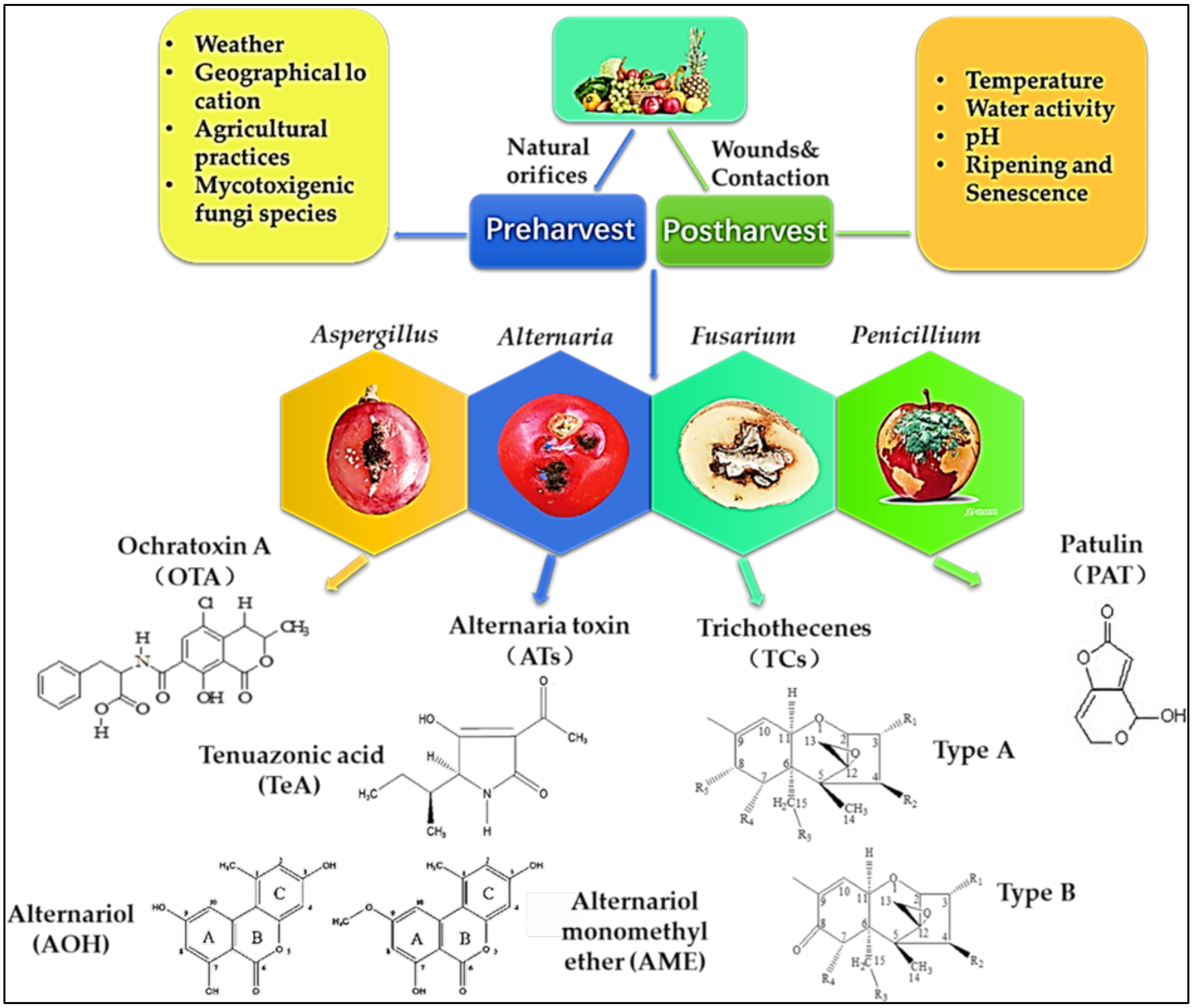

Figure 2.1: Mycotoxin chemical structures and conditions that allow fungal and mycotoxin production in fruits and vegetables (Nan, Xue and Bi, 2022).

Owing to their toxic nature, the detection of these substances is necessary. Several detection methods have been developed, including the chromatographic techniques employed on a wide scale. The procedure for detecting mycotoxins involves extraction from the sample material and purification, followed by qualitative and quantitative analysis (Aiko & Mehta, 2015). Even though several mycotoxins have been detected in various commodities worldwide, those of keen interest in Africa and other tropical countries are aflatoxins (AF) and fumonisins (FB). There is available evidence suggesting that in addition to the AF (AFB1, B2, G1, and G2) and FB (FB1, FB2, and FB3), ochratoxin A (OTA) and the trichothecenes (TH) are also significant (Njobeh *et al*., 2012). Notably, the

Fusarium species are destructive plant pathogens producing mycotoxins before or immediately after harvest. Still, *Penicillium* and *Aspergillus* species commonly exist as contaminants of commodities and foods during drying and storage (Pitt *et al*., 2000).

Drying of foods is commonly practised in Africa to enhance the products' durability and preserve them for food-insecure periods (Aworh, 2021). The drying process is mainly conducted on an artisanal scale or through small-scale industrial units (Kirigia, Kasili and Mibus, 2017). The resultant dried products can be infected with fungi and other contaminants from the pathogens already present in the primary product or during the drying process associated with unhygienic conditions (Nafisa *et al*., 2017).

Reviewing the problem of dietary mycotoxins, Klich and colleagues (2003) remarked that the likelihood of mycotoxin exposure is more likely to occur in parts of the world where inadequate food handling and storage methods are common. Also, a few regulations protect the exposed populations (van der Walt *et al*., 2006). Mycotoxins still pose a severe threat to human and animal health across the globe. Knowing that the mycotoxins can be produced during both pre-harvesting and post-harvesting, the fight against the mycotoxins should commence in the field, preventing the entry of contaminated produce into the storage or processing facilities (Barkai-Golan and Paster, 2011).

2.11 Effects of mycotoxins on human and animal health

Mycotoxins are toxic compounds produced by particular moulds or fungi with some toxicity to living organisms (Atanda *et al*., 2011). Toxigenic fungi grow on different foods, including dried fruits and vegetables, cereals, nuts, and spices (Ozer, Basegmez and Ozay, 2012). On the other hand, mould can grow on food before and after harvest and during storage (Omotayo *et al*., 2019).

Humans can be directly exposed to mycotoxins by ingesting the foods mentioned in Table 2.5 and some animal products, resulting in diseases like mycotoxicosis, cerebral oedema and mycoses (Dharini *et al*., 2022). The susceptibility of humans to mycotoxins is influenced by age, gender, weight, diet, the number of toxins they are exposed to, and the presence of other mycotoxins in the same food (Afsah-Hejri *et al*., 2013). For instance, an infant is more vulnerable to mycotoxins than an adult. The health effects following invasion of mycotoxins can be categorised into acute or chronic mycotoxicosis (Awuchi *et al*., 2022). Consuming large amounts of toxin over a short period will cause acute toxicity leading to death, while small amounts over a long period will result in chronic effects to the consumer (Haque *et al*., 2020). In humans, acute form of mycotoxicosis might cause symptoms such as acute gastrointestinal illness accompanied by nausea, vomiting, diarrhoea, or headache (Placinta *et al*., 1999).

Other mycotoxins in food have been associated with long-term effects on health, including cancer and immune deficiency (WHO, 1989). Precisely, aflatoxins bind to the DNA and disrupt genetic coding, thus promoting carcinogenesis (Darwish *et al*., 2014). In 2004, there was a massive outbreak in Kenya, where 125 people died due to liver failure caused by acute aflatoxicosis after consuming contaminated maize (Aiko & Mehta, 2015). Complications usually ensue from mycotoxin contamination only when an individual suffers from a has an underlying disease (Kebede *et al*., 2020).

In animals, almost exposure to mycotoxins can be detrimental, however, the severity may vary from one animal species to the other as well as on the level and duration of the exposure (Yang, Song and Lim, 2020). In the Republic of South Africa, outbreaks of mycotoxicoses in animals have been reported due to consumption of contaminated feeds (Njobeh *et al*., 2012).

Table 2.5: Incidence of mycotoxins in agricultural crops and human diseases they may cause

Country	**Mycotoxin**	**Methods of detection**	**Foodstuffs**	**Effects**
South Africa	Fumonisins (FUMs)	Thin Layer chromatography (TLC), liquid chromatography (LC), liquid chromatography-mass spectrometry (LC-MS), gas chromatography-mass spectrometry (GC-MS), and high-performance liquid chromatography (HPLC).	Maize	Tumours of the kidney and liver
	Fumonisins (FUMs)	Thin layer chromatography (TLC), liquid chromatography (LC), liquid chromatography-mass spectrometry (LC-MS), gas chromatography-mass spectrometry (GC-MS), and high-performance liquid chromatography (HPLC)	Compound feeds	Cerebral oedema
	Deoxynivalenol (DON)	Lateral flow or dipstick immunoassay	Compound feeds	Immuno-depressants, gastrointestinal haemorrhaging
	Zearalenone (ZEA)	Lateral flow or dipstick immunoassay	Compound feeds	Estrogenic activity (infertility, vulval oedema, vaginal prolapse, mammary hypertrophy in females, feminization of males)
Lesotho	Zearalenone (ZEA)	Lateral flow or dipstick immunoassay	Sorghum beer	Estrogenic activity
USA	Aflatoxins (AFs)	Enzyme-linked immunosorbent assay (ELISA)	Maize, wheat, rice, sorghum, ground nuts, tree nuts, figs	Liver lesions, cirrhosis, primary hepatocellular carcinoma, Kwashiorkor, Reye's syndrome
	Ochratoxin A	High-performance liquid chromatography (HPLC)	Cereals, dried vine fruit, wine, coffee	Endemic nephropathy, urothelial tumours

(Njobeh et al., 2012; Reddy et al., 2008; Patey and Gilbert 1989)

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CHAPTER 3

TRADITIONAL LEAFY VEGETABLES SOLD IN THE FREE STATE, SOUTH AFRICA

3.1 Introduction

The extent of use of wild green leafy vegetables varies with climate, soil, and vegetation differences. It also varies with different lifestyles and indigenous traditions of other ethnic groups across Africa. Therefore, these variances reflect differences in the availability and use of edible plants (Vainio-Mattila, 2000). There is little information about the available markets where traditional leafy vegetables are sold in South Africa since vendors primarily sell them informally. The vegetables are traded in Central Business Districts (CBDs) to consumers (predominantly black people) as medicines or food. Most of these vegetables are collected from the wild and sold in informal urban markets (Faber *et al*., 2010). Vorster's (2008) study on the role and production of indigenous leafy vegetables in three South African rural communities demonstrates that traditional leafy vegetables are not widely marketed. Furthermore, Van Rensburg *et al*. (2004) revealed that the sale of fresh traditional leafy vegetables was low, occurring at only 3% of total TLVs sales. This finding is unsurprising as most people prefer to purchase dry vegetables, which are less perishable, can be easily stored for longer, and are easily transported.

Traditional leafy vegetables are sold in markets that are not well planned, added to the fact that their conditions are very unhygienic, without storage facilities and infrastructure. Furthermore, the high level of illiteracy and the lack of or the limited availability of business skills amongst the traders, the informal nature of the trade, and the knowledge that these vegetables are regarded as poor people's food have caused the industry to remain undeveloped and possibly never being developed (Mander, 1998). Despite this, Lewu and Mavengahama (2010) argue that these vegetables could become commercialised if further studies were conducted on their production.

Information on the trade of TLVs in the Free State region of South Africa is scarce. There is a lack of knowledge on the characteristics of the trade, the plant species available, their preservation and storage conditions. This study aimed to identify markets that sell TLVs used either as food or medicine in the Free State Province and to determine post-harvest and storage practices for these vegetables.

3.2 Methods

3.2.1 Study area

The study was conducted in selected towns in the Free State Province, South Africa. The province was estimated to have a population of 2.8 million in 2019 (Statistics SA, 2016). Its capital is Bloemfontein, which is situated at 28°S 27°E and is considered the judicial capital of South Africa. The Free State province has rich soil and a climate for booming crop production (Dube *et al*., 2019).

The research was carried out in the province's urban and rural areas, including Sasolburg, Parys, Kroonstad, Welkom, Bethlehem, Bultfontein, Senekal, Winburg, Boshof, Bloemfontein, Ladybrand, Boshof, Thaba Nchu, Dewetsdorp, Wepener, Zastron, Brandfort and Phuthaditjhaba.

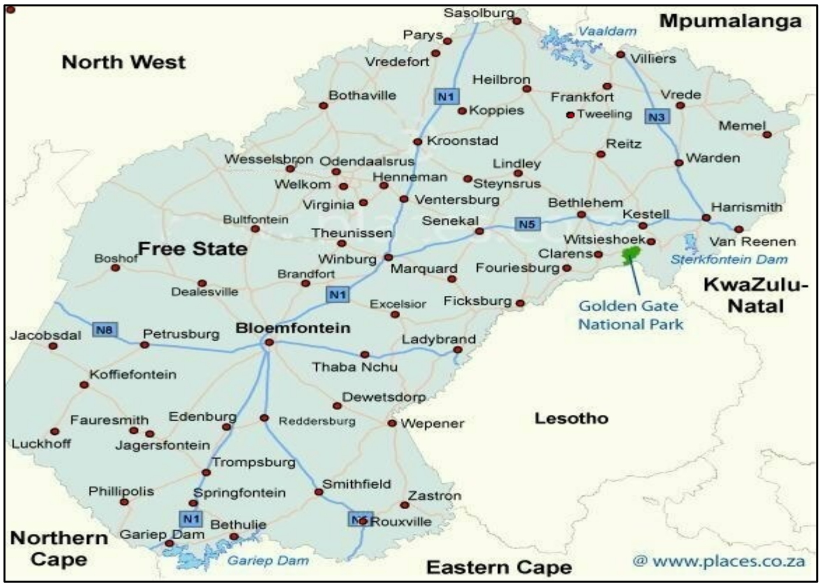

Figure 3.1: Map of the Free State showing various towns in the province (SA Places, 2022).

3.2.2 Data collection

Before collecting data, ethical clearance was approved by the Health Science Research Ethics Committee (HSREC), UFS. The Department of Health, Free State, also permitted the researcher to conduct the study. Informed consent was obtained from all the participants. In the light of this, every participant was informed of the nature of the study and allowed to choose whether to participate or decline. Following acceptance to participate, the participants were given consent forms describing the study and their right to withdraw their involvement whenever necessary. All the participants were given confidentiality concerning the information released into the study. Every research finding was not fabricated, but each was presented with integrity.

The research followed a descriptive cross-sectional survey design in which interview guides were used to interview the study population and collect data from the respondents. The target populations were the vendors that sold TLVs used either as food or medicines. The interviews consisted of individual, face-to-face verbal information exchange using semi-structured interviews and observations. This method was the most appropriate, allowing the researcher to control the order and flow of questions because most of the participants were illiterate. Generally, each interview was conducted in Sotho and lasted approximately 15-30 minutes. In total, 60 questionnaires were completed, from which data were recovered between October 2019 and March 2020.

Each interview questionnaire (Annexure D) consisted of five sections, namely; Section A: (location of the respondents); Section B: (personal information of the participants); Section C: (information about the business); Section D: (knowledge of traditional leafy vegetables); and Section E: (preservation of traditional leafy vegetables). Traders were questioned to obtain in-depth information about where the vegetables were collected, how they were stored and maintained, for how long preservation was done, and lastly, the preference of their customers, whether fresh or preserved vegetables. Plant names were provided in the local language and scientific names were identified from the literature.

3.2.3 Data analysis

All the quantitative descriptive data obtained from all the evaluations undertaken were recorded on a spreadsheet using Microsoft Excel 2007 and summarized using frequency tables, pie charts and bar charts.

3.3 Results and discussions

3.3.1 Demographic details of the traditional leafy vegetable traders

The market survey was conducted to generate and assemble information on the availability of TLVs in the Free State Province. Survey questionnaires were responded to by 60 participants, categorised into 55% males and 45% females. In a study by Buabeng and Osei-Kwarteng (2002), women were the ones more involved in the marketing of TLVs, whereas, in this study, men were the leading retailers. Notwithstanding, our findings are similar with those of Doibale *et al*. (2019), in which most vendors were male (79.2%), with only 20.8% females. Although women have always dominated this industry, studies have indicated an increase in men whenever crops gain value in the markets (Mahlangu, 2014). The involvement of men more than women in this business could prove advancement in commercialising TLVs.

Table 3.1: Demographic composition of the respondents

Variable	Features	Number	Percentage (%)
Gender	Male	33	55.00
	Female	27	45.00
Ages (years)	20-30	10	16.66
	30-40	20	33.33
	40-50	15	25.00
	50-60	10	16.66
	60-70	5	8.33
Ethnic groups/ origin of birth	Sotho	45	75.00
	Zulu	10	16.66
	Malawian	1	1.67
	Swati	2	3.33
	Ndebele	1	1.67
	Xhosa	1	1.67
Marital status	Single	17	28.33
	Married	43	71.67

Table 3.1 similarly illustrates the age distribution of the vendors of TLVs. The ages ranged between 20 and 70 years. However, most (33.33%) respondents fall into the 30-40 category, followed by those between 40 and 50 years, with 25% occurence. Many middle-aged people are involved in the trade of TLVs to survive and support their dependents.

All the respondents were of African descent, with a more significant number being Sotho (75%). Our data revealed that the Free State province has diverse ethnic groups, including Zulu (16.66%), Malawian (1.67%), Swati (3.33%), Ndebele (1.67%), and and the rest were Xhosa (1.67%).

3.3.2 Business information

Of the 60 respondents interviewed, 63.33% sold medicinal plants, 5% sold traditional leafy vegetables, 16.66% sold a combination of medicinal plants and traditional leafy vegetables, and 15% of the markets that were visited did not sell any traditional leafy vegetables. A large proportion (25%) of the respondents had been in the traditional plant business for about 20 years, while a minor proportion (8.33%) comprised traders who had been in business for nearly 30 years.

The market characteristics for the sale of plants by the target population are shown in Table 3.2. The survey revealed that 63.33% of the traditional leafy vegetables were sold for medicinal purposes and only 5% as traditional leafy vegetables. The low percentage of traditional leafy vegetables compared to medicinal plants may be ascribed to the loss of knowledge, processing, distribution, and marketing, as well as the nutritional information about these traditional food plants in conjunction with the fact that these traditional leafy vegetables are treated as weeds (Maseko *et al*., 2017).

Table 3.2: Business information of traditional leafy vegetable traders in Free State Province

Variables	**Features**	**Number**	**Percentage**
Merchandise sold	Medicinal plants	38	63.33
	Traditional leafy vegetables	3	5.00
	Both	10	16.66
	Neither	9	15.00
Existence of business	1-6 months	6	10.00
	1-10 years	15	25.00
	10-20 years	15	25.00
	20-30 years	5	8.33
	31+ years	10	16.66
	Non-existant	9	15.00
Purchase frequency of TLV	Weekly	4	6.66
	Monthly	1	1.66
	Rarely	38	63.33
	Whenever available	8	13.33
	Not sold	9	15.00

The low numbers of vendors selling TLVs may be due to urbanisation and lack of access to the vegetables. Following the introduction of exotic vegetables, TLVs have experienced a declining phase, as they are not cultivated but only collected from cultivated fields and the wild (Maseko *et al*., 2017). Traditional leafy vegetables are not as marketed as other vegetables or included in dietary plans. As a result, they are primarily sold in informal markets and rarely found in supermarkets. All these could significantly contribute to their reduced consumption.

3.3.3 Common traditional leafy vegetables sold in the Free State Province

Table 3.3 shows ten ethnospecies of TLVs belonging to Asteraceae, Urticaceae, Brassicaceae, Amaranthaceae, Campanulaceae, Chenopodiaceae, and Malvaceae families that were sold in the Free State markets (Bloemfontein CDB, Ladybrand CBD, Thaba-Nchu CBD, Welkom CBD, Phuthaditjhaba CBD, Sasolburg CBD, Parys CBD, Kroonstad CBD, Dewetsdorp CBD, and Zastron CBD). *Amaranthus* sp. and *Cucurbita maxima* found at a market in Bloemfontein CBD are shown in Figure 3.2.

Figure 3.2: Traditional leafy vegetables sold at one informal market in Bloemfontein, Free State. A; Amaranthus hybridus, B; Cucurbita maxima

The number of TLVs found in this region and identified in this study represent only a tenth of the hundred different species that have been documented from South Africa (Maseko *et al*., 2017; Mungofa, 2016). The most frequently purchased vegetable species included *Malva sp., Rorippa nudiuscula* Thell.*, Urtica sp., Lepidium bonariense* L.*, Sonchus dregeanus* DC.*, Amaranthus sp., Wahlenbergia undulatae* and *Lepidium capense* L. However, there is a variation in the number of vegetables documented in the different towns.

Table 3.3: List of traditional leafy vegtables sold in markets from selected towns in the Free State Province

Scientific name (Local name)	**Parts used**	**Nutritional/medicinal uses**	**Collection frequency**	**Collection site**	**Locations**	**Packaging**
Sonchus dregeanus DC. (Leharasoane)	Roots and leaves	Food and Medicine	Once or twice a month	Lesotho The veld Mountains	Bloemfontein Zastron Phuthaditjhaba	Polypropylene or Polyethylene bags
Urtica sp. (Bobatsi)	Leaves and stems	Leaves are cooked with onions, tomatoes and potatoes. Served with any starch food.	Once or twice a month	The veld The mountains of Lesotho	Zastron Phuthaditjhaba Parys Kroonstad	Newspapers Polypropylene bags Polyethylene bags
Rorippa nudiuscula Thell (Papasane)		Food and medicine	Twice a month	The veld Mountains	Zastron	Polyethylene bags
Wahlenbergia undulate (L.f) A.DC. (Tenane)	Leaves	Leaves are eaten like spinach, and plant decoctions are used to treat intestinal ulceration in children	Once or twice a month	Farms The natural veld	Dewetsdorp Bloemfontein Zastron	Polypropylene bags Polyethylene bags
Lepidium capense Thunb. (Qhela)	Roots and leaves	Food	Once or twice a month	The veld Mountains	Dewetsdorp Bloemfontein Zastron Welkom Phuthaditjhaba	Polypropylene bags Polyethylene bags Newspapers
Lepidium bonariense L. (Sebitsa)	Roots and leaves	Food and Medicine	Once a month	The veld Johannesburg	Kroonstad Zastron	Polyethylene bags
Amaranthus sp. (Theepe)	Leaves	Fresh leaves are cooked with onions and tomatoes and served with pap or bread.	Once or twice a month.	Farms Home gardens The veld	Dewetsdorp Ladybrand Bloemfontein	Polyethylene bags
Malva sp. (Tikamotse)	Roots and leaves	Food and medicine	Once a month	Johannesburg	Sasolburg	Polypropylene bags Polyethylene bags
Chenopodium album L. (Seruoe)	Leaves	Fresh leaves are boiled and eaten	Twice a month	The veld Lesotho	Bloemfontein	Polyethylene bags

		alone or with pap, rice or bread.				
Cucurbita maxima (Lihaba / mokopu)	Leaves	Fresh leaves and young pumpkins are cooked and served with pap or rice.	Weekly	Home gardens	Bloemfontein Thaba-Nchu	Polyethylene bags

Other studies indicated that *Amaranthus sp., Cleome gynandra, Cucurbita maxima, Portulaca oleracea, Bidens pilosa, Corchorus olitorius, Solanum nigrum,* and *Momordica balsamina* are primarily utilised in other provinces such as Mpumalanga, Limpopo, and KwaZulu-Natal (Vorster *et al*., 2008) which might therefore explain the low usage of these vegetables in the Free State as there is every possibility that the vegetables are more distributed in other provinces than in others. This may be due to environmental factors such as precipitation and temperature. Most studies on TLVs in the literature are mainly from Mpumalanga, Limpopo, and KwaZulu-Natal provinces which have a tropical climate that favours the growth of TLVs.

3.3.4 Collection of traditional leafy vegetables

Traditional leafy vegetables can be collected from the veld, farm fields, or gardens. In this study, traders obtained the plants from; their own gardens, Lesotho, the veld, and Faraday Muti Market in Johannesburg (Figure 3.3). 47.1% of the plants were procured from Faraday Muti Market, 37.3% from the veld, 9.8% from Lesotho, and lastly, 5.9% of the traders' gardens.

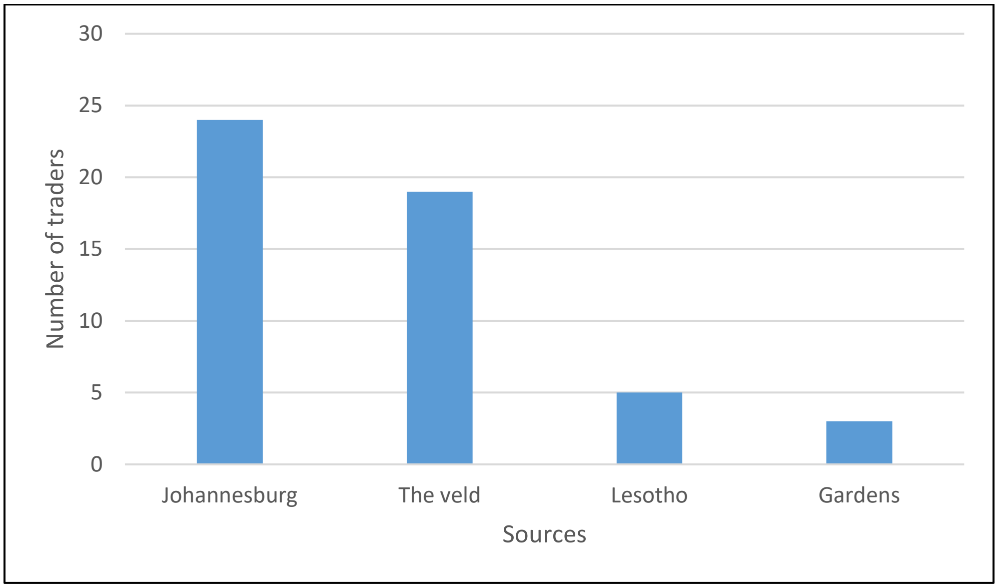

Figure 3.3: Sources (collection areas) of traditional leafy vegetables

According to van Rensburg *et al*. (2007), African people obtain TLVs in different ways; they may be harvested from the wild, cultivated fields or cultivated in gardens. Their findings are similar to part of our data, which indicates that TLVs are collected from the veld, cultivated fields, and own gardens. Most TLVs remain as wild species or weeds; hence they are not cultivated. However, the few cultivated include the *Cucurbita maxima, Amaranthus hybridus* L., *Abelmoschus esculentus (L.) Moench var. esculentus*, *Brassica rapa* L., *Citrullus lanatus* Thunb., *Cucumis melo* L., *Solanum retroflexum*, and *Vigna unguiculata* L. (Maseko *et al*., 2017) consequently, leading to a decline in the utilisation of these vegetables.

3.3.5 Storage of the traditional leafy vegetables

The majority of the TLVs sold in the informal markets in the Free State were stored in polyethylene bags (41%), a few were not packaged (8%), 37% of the plants were stored in polypropylene bags (empty maize meal bags) after collection, while 14% were folded in newspapers (Figure 3.4).

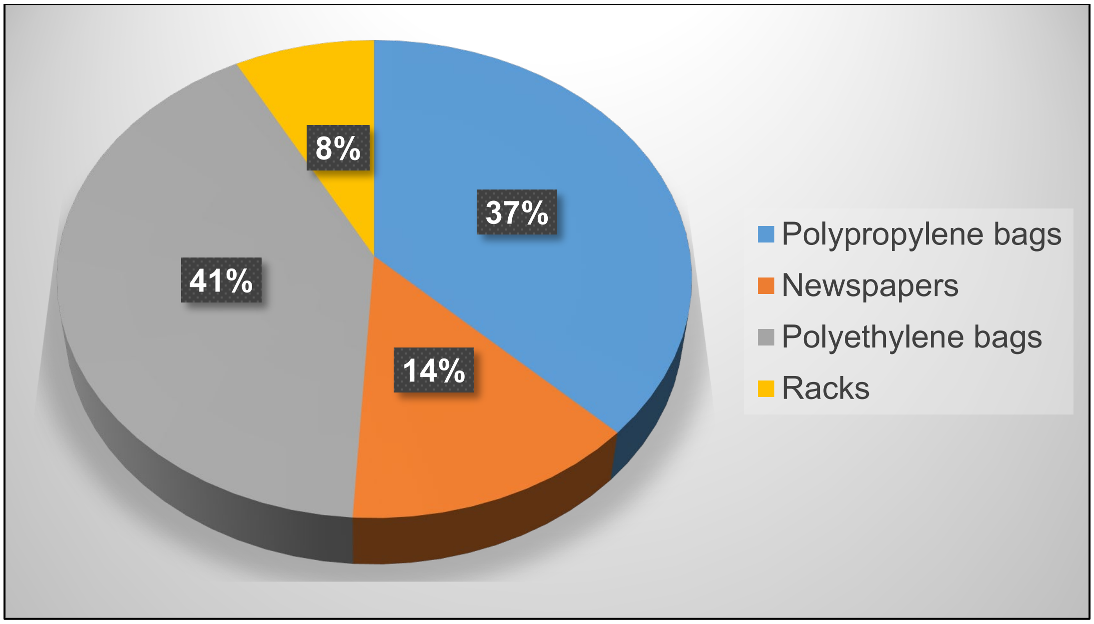

Figure 3.4: Pie chart showing the storage profiles of traditional leafy vegetables from Free State

Figure 3.4: Pie chart showing the storage profiles of traditional leafy vegetables from Free State

As depicted in Figure 3.4, most traditional plant traders preferred using polyethylene bags for storage or trading their merchandise because they are cheap and easily obtainable. Similarly, Vorster *et al*. (2008) noted that dried TLVs are pre-packaged and sold in polyethylene bags. Traditional leafy vegetables are known for their short fresh shelf life, therefore, necessitating the drying of the plants. However, the storage conditions of any processed goods are vital in maintaining conditions that inhibit the growth of microbes. Therefore, storing these plants in plastic bags may not be ideal. Fungi, for example, have optimal growth in an environment with a temperature of 30°C; thus, if dehydrated vegetables are kept at room temperature in a range of 25-30°C, this environmental condition may favour optimum fungal growth (Akeredolu and Adebajo, 2013). It is highly imperative, therefore, to verify the environmental conditions in the storage areas. While traditional methods of storage may be effective in preserving the integrity of TLVs, these are usually applied at household level and may not be appropriate in a market setting. For example, van Rensburg and colleagues (2004) reported the storage of TLVs in clay pots or in polypropylene sacks. The pots are kept in the kitchen close to a fireplace, with the belief that the smoke from the fire would hinder mould development on the dried vegetables. On the other hand, the sack is kept on top of an object or hung to avoid touching the ground to limit microbial contamination.

3.3.6 Preservation of traditional leafy vegetables

The findings from the survey showed that 96% of the plant species involved were preserved by drying then stored for future use, whereas 4% were not preserved. The interviewees mentioned that they preserved the vegetables by removing the soil from the plants after harvesting and placing them directly in the sun for drying until they were dehydrated (49%). In addition, 47% of them are obtained already dried vegetables from other suppliers. The participants mentioned that they spread the vegetables on flat surfaces in the open to dry them.

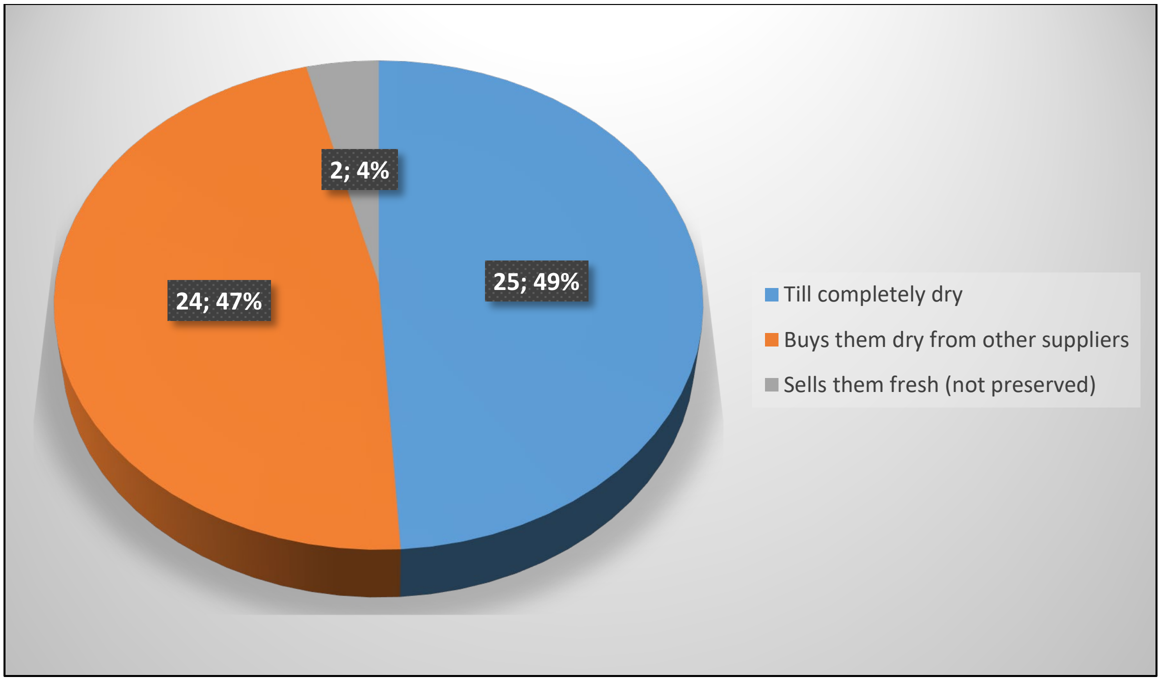

Figure 3.5: Different preservation methods of traditional leafy vegetables from Free State province

Most traditional leafy vegetables are seasonal and highly perishable (Mepba, Eboh and Banigo, 2007; van Rensburg *et al*., 2007). Therefore, preservation becomes crucial to ensure that the vegetables are available, especially for dry seasons when there is scarcity, leaving many underprivileged families hungry (Kiremire, 2010; Lewu and Mavenghama, 2010).

In addition, through preservation, the biological activity is reduced to a level that compromises microbial growth (Indiarto *et al*., 2021). This may also compromise the consumer's health (Demarchi *et al*., 2013) as the preservation methods, including salting and sun drying, boiling and sun drying, sun drying causes a reduction in micronutrients (between 20 and 82%) in TLVs (Faber *et al*., 2010; Bighagfire *et al*., 2021). The sun also causes the discolourisation of the vegetables (Tembo *et al*., 2008) which was observed among the vegetable samples purchased from the markets for this study.

Rural households employ sun-drying, a technique associated with indigenous knowledge systems, to address the problem of seasonal shortages of vegetables and other foods since they have limited resources (Nyembe, 2015; Faber *et al*., 2010). Local people have a rich knowledge of these techniques due to their routine practices, built with experience over time (van Rensburg *et al*., 2004). In South Africa, sun drying is the predominant method of preserving leafy vegetables and dried vegetables constitute a significant part (80%) of the food rural households consumed during the winter (Vorster *et al*., 2008). Aside from sun-drying, households can employ other preservation technologies, including solar drying, oven-drying, freezing, canning, or bottling (Nyembe, 2015).

3.4 Conclusion

The results of this study show that TLVs are not entirely neglected by communities in the Free State Province, even though there has been a decline in utilization over the years. The introduction of exotic vegetables, urbanization, lack of knowledge imparting TLVs to the youth and referring to TLVs as poverty foods are some reasons for the decreased utilization of these vegetables. Most of these vegetables are collected from the wild hence their inaccessibility by most people; however, some, such as *Amaranthus hybridus* L. and *Cucurbita maxima*, can be cultivated and introduced to formal markets. There is a need to include TLVs in the South African diet to address issues like malnutrition and food insecurity. Moreover, people need to be educated about the health benefits of traditional vegetables to change their perception towards them.

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CHAPTER 4

CHARACTERISATION AND IDENTIFICATION OF FUNGAL CONTAMINANTS IN SELECTED TRADITIONAL LEAFY VEGETABLES

4.1 Introduction

Traditional leafy vegetables (TLVs), consisting of *Amaranthus hybridus* L.*, Cleome gynandra* L.*, Cucurbita maxima* and *Lepidium capense* Thunb, are essential to a healthy diet (Sarkar, *et al*., 2022). They play a vital role in food security by alleviating hunger and malnutrition (Van Rensburg, 2004). They are an excellent source of proteins, vitamins, minerals, carotenoids and polyphenols (Opazo-Navarrete *et al*., 2021; Odhav *et al*., 2007). In addition to their nutritional value, TLVs provides an economic opportunity that reduces poverty and unemployment for smallholder farmers in rural areas (Schreinemachers *et al*., 2018). Vegetables such as amaranth, jute mallow and African nightshade can be sold in formal and informal markets, thus contributing to household income (Ebert, 2014). Traditional leafy vegetables are readily available and gathered, primarily by women and children from farmers' fields and gardens, the wild, and very few are cultivated (Masarirambi *et al*., 2020). They do not require much to be cultivated as they can be grown in areas with low, unreliable rainfall, steep topography and poor soils (Mathaba, 2017).

Traditional leafy vegetables are handled and stored briefly before being sold in informal markets. Vegetables in excess are typically sun-dried, solar-dried or canned to prolong their shelf life (Masarirambi *et al*., 2010). However, this creates an environment susceptible to contamination with dust, insects, bacteria and fungi (Masarirambi *et al*., 2020). According to Yaradua *et al*. (2018), 20% of vegetables meant for human consumption are affected by microbial spoilage. Microbial pathogens like bacteria, yeasts and fungi infect many plants, including TLVs, under different conditions (Yaradua *et al*., 2018). The vegetables may be contaminated during processing, storage, marketing practices, transportation, and climatic factors, leading to spoilage and fungal contamination (Chen, 2020; Toma and Abdulla, 2013).

It is, therefore, imperative to assess, identify and characterise fungal contaminants to evaluate the quality of TLVs and develop methods to control or minimise contamination (Ikenna, 2020). Various studies have been conducted on the importance, uses, consumption intensity and other aspects of traditional leafy vegetables, but very few have focused on their contamination (Imathiu, 2021; Gido *et al*., 2017; Randhawa *et al*., 2015). Therefore, an investigation into the fungal contamination of TLVs becomes very crucial. Mycotoxigenic fungi and the mycotoxins they produce have toxic effects such as neurotoxicity, carcinogenicity, and immunosuppression in humans and animals (Rahman *et al*., 2019). This study evaluated the characterization and identification of mycotoxigenic fungi in TLVs and the prevalence of fungal contamination.

4.2 Methods

4.2.1 Collection of samples

Five TLVs listed in Table 4.1 were purchased from the traditional healers and street vendors in selected towns in the Free State province. The vegetables were collected based on their availability during the market survey and their popularity of usage. Most of the traditional leafy vegetables purchased were displayed in plastic bags, polypropylene bags and newspapers on the ground in informal street markets (Table 4.1). Subsequently, the vegetables samples were transferred into sterile polyethylene bags and transported to the Central University of Technology in a cooler box to prevent possible contamination, to the CAFSaB microbiology laboratory for fungal analysis.

Table 4.1: Profiles of selected traditional leafy samples from selected areas of Free State

Scientific name (Local name)	Sample numbers	Collection sites	Condition when purchased	Market displaying material
Cucurbita maxima (Mokopu)	CmThN1 CmBL1	Thaba-Nchu, Bloemfontetin	Fresh	Plastic bags
Chenopodium album L. (Seruoe)	CaQw1 CaThN1	Qwa-Qwa Thaba-Nchu	Dried	Newspapers
Rorippa nudiuscula Thell. (Papasane)	RnuBL1	Bloemfontein	Dried	Polypropylene bags
Amaranthus hybridus L. subsp. *hybridus* var. *hybridus*(Theepe)	AmaThN1 AmaBL1 AmaQw1	Thaba-Nchu Bloemfontein Qwa-Qwa	Fresh	Plastic bags
Lepidium capense Thunb. (Qhela)	LepThN1	Thaba-Nchu	Dried	Newspapers

4.2.2 Sample preparation

The sun-dried and fresh vegetables were collected. The dried vegetable samples were kept in a refrigerator (4ºC) till fungal and mycotoxin analysis, whereas the fresh ones were rinsed in sterile distilled water and dried in a laminar air flow dyer (Lasec, South Africa). The samples were then individually ground to a powder using a Kinematica Polymix PX-MFC 90 D Laboratory Grinding Mill (Produced by Kinematica AG, Switzerland) at 302.1rpm (revolutions per minute). The grinding mill was rinsed with 96 % ethanol before and between samples to minimise contamination. Each powdered plant sample was kept in separate sterile polyethylene bags and stored at room temperature for further analysis. (Hell *et al*., 2009).

4.2.3 Isolation and production of purified fungal cultures

4.2.3.1 Standard dilution plating

The serial dilution method was used to determine total fungal counts in the vegetable samples. One gram (1g) of each powdered vegetable was weighed and dispensed into 9mL of distilled water and homogenized using a Vortex-Genie mixer (Scientific Industries, Inc, United States). Five serial dilutions (10^{-1} – 10^{-5}) for each plant sample were prepared, thus involving five (5) sterile test tubes, each containing 9 mL of distilled water. Accordingly, 1mL of the stock was transferred from the first tube (10^{-1}) to the next and, subsequently, to all the remaining sterile test tubes till the last tube (10^{-5}) while shaking after each transfer to ensure complete and uniform mixing of the content. This process produces 10mL of the diluted solution. After that, a small volume of each dilution is used to make a series of spread plates. (Nayak, Choudhary and MG, 2022).

4.2.3.2 Isolation media

Different microbiological media were employed for the cultivation of fungal cells. They entailed the following:

a) Malt extract agar

Malt extract agar (MEA) was used to isolate fungi from TLVs samples. The medium was prepared following the manufacturer’s instructions. Fifty grams (50_g) of MEA was weighed and suspended in 1_L of distilled water. Chloramphenicol (0.01_g) was added to the agar medium to prevent bacterial growth. The contents were thoroughly mixed using a magnetic stirrer to ensure complete dissolution of the agar powder. The medium was then sterilized by autoclaving at 121◦C for 15_min. Later, the medium was allowed to cool to 45-50◦C in the water bath (Lasec, South Africa) for 5_min. The medium was dispensed into 90_mm sterile petri dishes (Abdos Lifesciences, India), and allowed to solidify.

b) Potato Dextrose Agar

Potato dextrose agar (PDA) was used for sub-culturing fungi obtained from the malt extract agar. During this procedure, fungal growth on cultured plates or stock culture is transferred onto freshly prepared medium to evade contamination and enhance cell growth's purity. Similarly, 39_g of PDA powder was weighed and dissolved in 1_L of demineralized water. Approximately 0.01_g of chloramphenicol was added to the agar medium to prevent bacterial growth. The nutritive medium was then autoclaved at 121°C for 15_min. Finally, the agar was cooled to 45-50°C and poured into 65_mm sterile petri dishes (Abdos LifeSciences, India), and allowed to solidify.

Two methods were used to isolate fungi from the vegetable samples: direct plating and dilution plating. In the direct plating method, 0.3_g of the plant powder was sprinkled on the surface of the agar plate, whereas, in dilution plating, 1_mL of a serially diluted specimen was pipetted onto the centre of the surface of a 90mm plate containing freshly prepared malt extract agar (MEA). A sterile spreader was employed to evenly spread the sample over the agar surface while carefully rotating the petri dish and observing aseptic conditions. The plates were closed, sealed with parafilm and incubated at room temperature for 2 to 5 days. Each plate was examined daily for fungal growth. The observed fungal colonies were identified according to the morphological characteristics and were subcultured to obtain pure cultures (ICFM, 2007).

4.2.4 Enumeration of fungal counts (Colony counting) and sub-culturing

Following 2 to 5 days of incubation, fungal colonies were counted and recorded using the aCOLade colony counter (Symbiosis, United Kingdom), expressing the counts as colony forming unit per gram (CFU/g). For purity, fungal isolates were double hyphal tipped and subcultured onto freshly prepared PDA plates for further identification since they exhibited different characteristics in terms of colour and shape. While observing aseptic conditions, a sterile cork borer was used to cut 1 cm plugs from the edge of the fungal culture and placed them on PDA media. The plates were then closed, sealed with parafilm, and incubated for 48-72 hours at room temperature. The pure strains of fungi were identified using fungal identification keys (Klich, 2002; Domsch *et al*., 1993).

4.2.5 Storage of fungal cultures

After obtaining pure cultures, a few pieces of agar with mycelia were stored in sterile distilled water (referred to as Castellani’s method), and a single fraction was maintained on PDA slants in McCartney bottles (Chem Lab Supplies, South Africa) and kept at 4°C (Saxena and Gupta, 2019).

4.2.6 Genetic identification of fungal isolates

4.2.6.1 DNA extraction

Genomic deoxyribonucleic acid (DNA) was extracted from the cultures grown on PDA using the Quick-DNA Fungal/Bacterial Miniprep Kit (Zymo Research, Carlifornia, United States of America) according to the manufacturer's instructions. Fungal cells (50-100_mg) were added to 200_µL) of water contained in the ZR Bashing lysis tube with 750_µL of Bashing bead buffer. It was then centrifuged at maximum speed for ≥5 min. The ZR Bashing Bead lysis tube was centrifuged (Lasec, South Africa) at 10 000×g for 1_min. Subsequently, 400_µL of the supernatant was transferred to a Zymo-Spin III-F in a collection tube and centrifuged at 8 000×g for a minute. In addition, 1 200_µL of genomic lysis buffer was introduced into the filtrate in the collection tube. Later, 800_µL of the mixture was transferred to a Zymo-Spin IICR column in the collection tube and centrifuged at 10 000×g for 1_min. This step was repeated after discarding the flow-through before adding 200_µL of DNA pre-wash buffer to the Zymo Spin IICR Column in a new collection tube and centrifuging at 10 000×g for a minute.

After centrifugation, 500_µL of g-DNA wash buffer was added to the Zymo-Spin IICR column and centrifuged at 10 000×g for 1_min. At the final stage, the Zymo-Spin IICR column was transferred to a clean 1.5_mL microcentrifuge tube, and 100_µL of DNA elution buffer was added directly to the column matrix. The mixture was centrifuged at 10 000×g for 30_sec to elute the DNA (William *et al*., 2012).

4.2.6.2 Polymerase chain reaction (PCR) amplification

Two sets of primers listed in **Table 4.2** were used to amplify the target genes following the procedure previously described by White and colleagues (1990). The internal transcribed spacer (ITS) region, which is the preferred DNA barcode for fungi, was amplified using OneTaq Quick-Load 2X Master Mix in a total reaction volume of 50_µL, which was constituted based on the following:

- OneTaq Quick-Load 2X Master Mix with Standard Buffer,
- 10_µM forward primer,
- 10_µM reverse primer,
- 1_ng-1_µg genomic DNA;
- and nuclease-free water.

The components were assembled on ice and quickly transferred to a thermocycler (ThermoFisher Scientific, South Africa). The amplification conditions constituted an initial denaturation of 30_sec at 94°C, followed by 30 cycles of denaturation for 15-60_sec at 45-68°C and a final extension of 5_min at 68°C. The PCR products were electrophoresed on a gel, and the amplified DNA contained in the gel was extracted with the Zymoclean Gel DNA Recovery Kit (Zymo Research, California, United States of America).

Table 4.2: ITS Primers sequences

Name of Primer (and region)	Target	Sequence (5' to 3')	Reference
ITS1	Small Sub-Unit	TCCGTAGGTGAACCTGCGG	White *et al*., 1990
ITS4	Large Sub-Unit	TCCTCCGCTTATTGATATGC	White *et al*., 1990

4.2.6.3 DNA sequencing and purification

The amplified fragments were sequenced in the forward and reverse direction using Nimagen, Brilliant Dye Terminator Cycle sequencing Kit (V3.1, BRD3-100/1000). The kit is based on the Sanger Chain Termination method and has the following content: Reaction Ready Seq. premix, 5Xsequencing buffer, pGem control and M_{13} (-21) primer.

Briefly, the amplicons were purified using the ZR-96 DNA sequencing clean-up kit and analysed on the ABI 3500xL genetic analyzer (DL Biotech Co., China) for each reaction of every sample. The CLC Bio Main Workbench v7.6 was used to analyze the ab1 files generated by the ABI 3500xL Genetic Analyser. A basic local alignment search tool (BLAST) was used to obtain the results via comparison with the already identified fungal isolates stored in the National Center for Biotechnology Information (NCBI) database.

4.3 Results and Discussion

4.3.1 Isolation and morphological identification of fungi

All the vegetables involved in this study were contaminated with fungi due to the presence of growth of the colonies, and the entire colonies varied with each sample. Colonies were counted; the vegetable sample with the highest count was *Cucurbita maxima* at 129 cfu/g while the *Chenopodium album* had the lowest at 19 cfu/g. The fungal colonies showed different morphological and cultural characteristics in colour, form, size and elevation, as shown in Figure 4.1. It can be depicted from Figure 4.1 that some plates had single colonies, whereas some had more than one colony or more colonies with different characteristics. The variation in fungal contaminants observed among the traditional leafy vegetables may be due to the various collection sites, the processing method, and the storage practices.

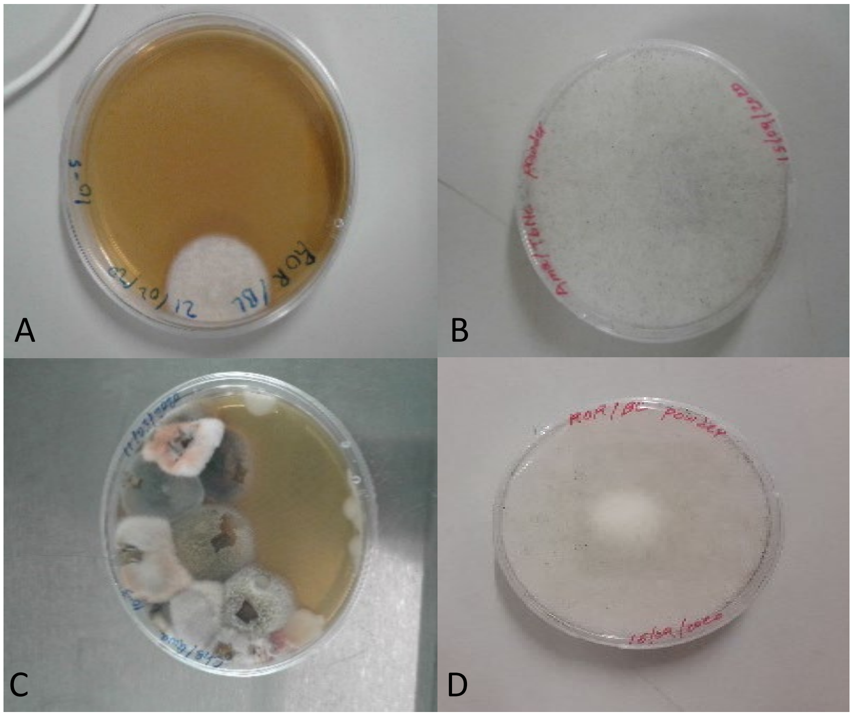

Figure 4.1: Morphological appearance of the different fungal colonies on MEA obtained from traditional leafy vegetable

The presence of fungi in the vegetable samples may have originated from the field or in storage facilities. The colonies showed white, greyish, greenish-black and black mycelium. Some moulds were distributed throughout the entire plate (Figure 4.1B), whereas some grew partially in different shapes and colors (Figure 4.1C). The greenish-black colony morphology resembled *Alternaria alternata*, the greyish colonies closely resembled *Rhizopus stolonifer*, and finally, the black ones resembled *Aspergillus niger* (Alsohaili & Bani-Hasan, 2018).

4.3.2 Molecular identification of fungal isolates

The amplified products were purified and sequenced, and the resulting sequences were employed in a BLASTn search for identity. They were compared to available already identified sequences stored in the NCBI database. Results from the BLAST prediction showed the presence of 9 fungal species, which were closely related to the following:

Epicoccum sorghinum, Alternaria alternata, Phoma sp, Cladosporium sp., Rhizopus oryzae, Nothophoma quercina, Fusarium sp., Didymella microstoma and *Didymella glomerata. Epicoccum sorghinum* and *Rhizopus oryzae* were the most prominent fungi (33.33%), followed by *Phoma sp*. (22.22%). Lastly, *Alternaria alternata*, *Cladosporium sp, Nothophoma quercina, Fusarium sp, Didymella macrostoma and Didymella glomerata* were (11.11%).

Table 4.3: Identified fungi isolated from the traditional leafy vegetable samples

Sample codes	**Place of collection**	**Scientific names**	**Colony forming units (cfu/g)**	**Identified fungi**
Ama-TbNc-10[1]	Thaba-Nchu	*Amaranthus hybridus* L. subsp. *hybridus* var. *hybridus*	30	*Epicoccum sorghinum*
Che-Qwa-10[2]	Qwa-Qwa	*Chenopodium album* L.	11	*Rhizopus oryzae*
Che-Qwa-10[3A]	Qwa-Qwa	*Chenopodium album* L.	1	*Epicoccum sorghinum*
Che-Qwa-10[3B]	Qwa-Qwa	*Chenopodium album* L.	2	*Alternaria alternata*
Che-Qwa-10[4]	*Qwa-Qwa*	*Chenopodium album* L.	3	*Phoma sp*., *Didymella sp.*
Che-TbNc-10[5]	*Thaba-Nchu*	*Chenopodium album* L.	1	*Cladosporium sp.*
Cur-BL-10[2]	Bloemfontein	*Cucurbita maxima*	36	*Rhizopus oryzae*
Cur-BL-10[4A]	Bloemfontein	*Cucurbita maxima*	1	*Nothophoma quercina*
Cur-BL-10[4B]	Bloemfontein	*Cucurbita maxima*	2	*Didymella microstoma*, *Didymella glomerata*, *Phoma sp.*,
Cur-BL-powder	Bloemfontein	*Cucurbita maxima*	90	*Fusarium sp.*,
ROR-BL-powder	Bloemfontein	*Rorippa nudiuscula* Thell.	27	*Rhizopus oryzae*
Che-Qwa-10[3C]	Qwa-Qwa	*Chenopodium album* L.	1	*Epicoccum sorghinum*

Table 4.3 shows that the traditional leafy vegetable, *Chenopodium album* L., was the most contaminated, harbouring diverse fungal species, including *Epicoccum sorghinum*, *Alternaria alternata*, *Phoma sp. or Didymella sp.* and *Cladosporium sp.* Similarly, *Cucurbita maxima* were the second vegetable with a high prevalence of fungal contaminants, including *Rhizopus oryzae, Nothophoma quercina, Fusarium sp, Didymella microstoma* or *Didymella glomerata* or *Phoma sp*. The findings revealed the

presence of nine (9) different fungal species, which is in agreement with those of Shehu *et al*. (2014), who noted the occurrence of 10 other fungal species from leafy vegetables from Sokoto Metropolis, Nigeria. Some fungi can affect various vegetables, causing devastating economic losses (Hua *et al*., 2018). They can enter the plant tissue via mechanical or chilling damage or following other organisms' deterioration of the skin barrier (Mailafia *et al*., 2017).

Fungi have been known for thousands of years to be ubiquitous, with spores that can travel vast distances across the surface of the Earth. A lot of agricultural products are attractive colonisation sites for fungi. Moreover, Hell *et al*. (2009) pointed out that fungi can develop directly on the surface of vegetables and even infect inner tissues. Microbial contamination of foods is very significant as it causes a reduction in the quality of the vegetable available for human consumption and the production of toxins, representing a health risk. According to the data presented in Table 4.4, the fungi species identified from traditional leafy vegetable samples belonged to nine species identified. Still, only *Fusarium, Alternaria alternata* and *Epicoccum sorghinum* can produce mycotoxins. Mycotoxins are secondary metabolites secreted by specific fungi and are known as potential top-priority agents causing human health hazards, ranging from minor to severe ailments affecting the skin, blood cells and liver resulting in leukopenia, immunodeficiency, skin necrosis and liver cancer. Also, the mycotoxins are non-protein in nature, thus offering resistance to heat and might compromise the health condition of the individual consuming foods contaminated with the metabolites (Perdoncini *et al*., 2019; Peromingo *et al*., 2019).

Fusarium species are plant pathogens affecting many plants and are responsible for contamination pre-harvest and harvesting (Greeff-Laubscher *et al*., 2020). In addition*,* *Epicoccum sorghinum* is a fungal contaminant commonly found in cereals such as sorghum during the pre-harvest stage, when the plant is fertilized with manure, sewage sludge and irrigated water and post-harvest stages. It produces a mycotoxin called tenuazonic acid (TeA), known for causing acute animal toxicity and a human hemorrhagic disorder (de Oliveira *et al*., 2018). Contamination of *Amaranthus hybridus* L. and

Chenopodium album L. with this fungus might have been possible because they grew in the same field. It has been established that the vegetables were collected in farm fields where cereals are being produced.

Another fungus related to food poisoning is *Alternaria alternata*, identified from one of the vegetables in this study (*Chenopodium album* L.). The fungus has been reported to infect fruits, vegetables and other agricultural products. It produces mycotoxins, including alternariol, altertoxins and L-tenuazonic acid (Troncoso-Rojas and Tiznado-Hernández, 2014). Considering that the fungus is found in the soil, it might have developed during the growing stages of the vegetable. Contamination could also be due to dust pollution as some dried vegetables were placed and spread on bare ground in open markets, where there is a lot of movement of people and vehicles (Sani *et al*., 2018).

According to Shehu *et al*. (2014), contamination of vegetables can result from injuries, high temperatures and storage in contaminated bins. However, it was observed in this study that some of the vegetables were stored for a long time in containers, thus increasing the moisture content and rendering them more susceptible to fungal growth, which could be avoided by following the seven principles of HACCP during the food production process, from raw material to finished products. The steps include conducting a hazard analysis, determining the critical control points, establishing critical limits, monitoring procedures, corrective actions, verification procedures, and record-keeping and documentation procedures.

4.4 Conclusion

From the results obtained in this study, it can be concluded that the dried traditional leafy vegetables sold in the informal markets in the Free State province of South Africa were contaminated with nine fungal species; *Epicoccum sorghinum, Alternaria alternata, Phoma sp, Cladosporium sp., Rhizopus oryzae, Nothophoma quercina, Fusarium sp., Didymella macrostoma* and *Didymella glomerata*, with four of them having the potential to produce mycotoxins (*Fusarium, Alternaria alternata, Epicoccum sorghinum, and Phoma sp*.). The mycotoxins produced by these fungi include tenuazonic acid, fumonisins, zearalenone, trichothecenes and monomethyl ether. With regards to the information obtained from the survey, the vegetables were exposed to environmental conditions that could favour fungal development and, consequently, mycotoxin production. Therefore, evaluating the risks associated with fungal and mycotoxin contamination in African populations is imperative, as this aspect hasn’t been thoroughly explored. Notwithstanding, there is a need to reduce the level of fungal contamination in the analysed vegetables through appropriate measures, which might include appropriate production practices (improved processing, packaging), storage and careful handling practices by the vendors, education and training on fungal and mycotoxin contamination, and the health implications. Additionally, the vegetables should be subjected to scrupulous quality control inspection before being released to reach consumers.

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CHAPTER 5

IDENTIFICATION OF MYCOTOXINS IN SELECTED TRADITIONAL LEAFY VEGETABLES

5.1 Introduction

Studies have shown that TLVs are good sources of essential nutrients (Mungofa, 2016). These vegetables contain nutrients such as vitamins A and C, iron, folate, zinc, calcium and magnesium (Uusika *et al*., 2010) and are dried to extend their shelf life in order to be used during the off-season (Nafisa *et al*., 2017). It is during the drying process where vegetables are susceptible to contamination as they are subjected to environments that favor fungal and mycotoxin contamination, for instance, the moisture content, temperature levels and pH levels (Akeredolu & Adebajo, 2013). More elaborately, fungi demonstrate a substantial role in the spoilage of vegetables; worse still, some of them are capable of producing mycotoxins through their different stages of pathogenesis in the harvested products (Barkai-Golan & Paster, 2008). Although fungi in foodstuffs are not often linked with mycotoxin production, the effect of fungal contamination is increased because mycotoxins might be present in the foodstuff, and there is no fungal growth present because of its stability. Moreover, a fungus can generate different mycotoxins, and various fungi can produce a mycotoxin (Fernández-Cruz *et al*., 2010).

The presence of microorganisms in plants is a problem experienced by many countries worldwide, especially with contamination resulting from fungi. Previous studies have indicated that some fungi species of the soil and field crops may contaminate traditional plants/medicine (van der Walt *et al*., 2006). Additionally, plants are contaminated with mycotoxins at any production stage, produced by toxigenic fungi under favourable environmental conditions. These mycotoxins cause many health implications, including carcinogenesis, teratogenesis, immune system suppression and mutagenesis (Zhang *et al*., 2018).

More than 25% of the world's agricultural products are mainly contaminated by mycotoxins (Ayalew *et al*., 2006). In the same way, the Food and Agriculture Organization

(FAO) has reported that relatively 25% of crops worldwide can be contaminated by mycotoxins (Luo *et al*., 2018). Approximately four hundred mycotoxins have been discovered, and those of most interest include aflatoxins, fumonisins, deoxynivalenol, trichothecenes, ochratoxin A, zearalenone and Alternaria toxins. In humans, zearalenone can cause cervical and breast cancer (Boermans and Leung, 2007; Abid-Essefi *et al*., 2004). It is interesting to note that, not all mycotoxins are produced by every living fungal species; however, mycotoxin production can be affected by several factors, including vegetable type, pre-harvest treatment, harvesting methods, climatic conditions, storage conditions and postharvest treatments (Barkai-Golan & Paster, 2008).

Mycotoxins occur naturally and not only do they affect human health and the quality of food, but their negative impact on human health also impacts the economy, resulting in economic losses for exporting countries (Moretti *et al*., 2013). Vegetables are no exception when it comes to the occurrence of mycotoxins, and this has been validated in many countries. Once mycotoxins have been produced in plant products, it is hard to eradicate them since they are small and stable (Jard *et al*., 2011). People in rural sub-Saharan Africa are more likely to be affected by chronic dietary mycotoxin exposure since their staple diets are mainly contaminated by mycotoxigenic fungi (Warth *et al*., 2012).

Some of the most common mycotoxins include the following; aflatoxins, ochratoxins, citrinin, patulin, deoxynivalenol, trichothecenes, sterigmatocytins, fumonisins, and zearalenone, which can contaminate plants pre-harvest, during harvest and/or post-harvest (Awuchi *et al*., 2021). Collectively these mycotoxins can have carcinogenic, mutagenic and other toxic effects on both animals and human beings (Altyn and Twaruzek, 2020). One of the significant strategies of solving the problem of mycotoxins is by implementing Good Agricultural Practices (GAP) and Good Manufacturing Practices (GMP) (Awuchi *et al*, 2021; Altyn and Twaruzek, 2020). Traditional leafy vegetables do not always meet quality and safety standards and the risk of mycotoxin contamination is a concern, hence the need to get them tested.

5.2 Materials and methods

5.2.1 Plant samples

The following plants, *Malva parviflora* L. var*. parviflora*, *Lepidium capense* Thunb., *Urtica dioica* L., *Amaranthus hybridus* L., *Chenopodium album* L., *Cucurbita maxima* and *Rorippa nudiuscula* Thell. were evaluated for the presence of 8 mycotoxins. The mycotoxins included aflatoxin B1, deoxynivalenol, nivalenol, ochratoxin A, zearalenone, fumonisin B1, fumonisin B2 and fumonisin B3. The table 5.1 below shows information about the plant samples analysed for the occurrence of the mycotoxins mentioned above.

Table 5.1: Information on the plant samples investigated for mycotoxin analysis

Scientific names	Local traditional names	State of vegetables when purchased
Malva parviflora L. var. *parviflora*	Tika Motse	Dry
Lepidium capense Thunb.	Qhela	Dry
Urtica dioica L.	Bobatsi	Dry
Amaranthus *hybridus* L. subsp. *hybridus* var. *hybridus*	Theepe	Fresh
Chenopodium album L.	Seruoe	Dry
Cucurbita sp.	Mokopu	Fresh
Rorippa nudiuscula Thell.	Papasane	Dry

5.2.2 Mycotoxin analysis

Approximately 5g (using three significant numbers) of the sample was weighed into a 50 mL tube. Twenty millilitres (20mL) of extraction solvent (50% water: 25% methanol: 25% acetonitrile) was added and sonicated (Qsonica, United State of America) for 1 hour. Subsequently, 1mL of the sample was dispensed into a 2mL Eppendorf tube (Eppendorf AG, Germany) and double diluted with the solvent, 75% water: 25% methanol. The contents in the tubes were centrifuged (Lasec, South Africa) for 5mins at 13 000rpm. Later, 1mL of diluted sample was pipetted into an analysis vial. A rapid and sensitive ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-ESI-MS/MS) was used to analyse and quantify the presence of mycotoxins in the vegetable samples (Spanjer *et al*., 2008).

5.3 Results and Discussion

Mycotoxins are toxic chemicals which are unavoidable contaminants, representing a significant challenge affecting food safety worldwide. They affect most foods and beverages during preparation, packaging, and transporting before consumption, and unfortunately, it involves a wide range of foodstuffs (Pakshir *et al*., 2020). The study was conducted to determine the presence of mycotoxins in dried and fresh vegetables that were marketed in the informal produce markets located in the Free State province. From Table 5.2**,** the mycotoxins identified were fumonisin B1 and fumonisin B2.

The two vegetable samples (*Chenopodium album* L*. and Amaranthus hybridus* L*.*) showed contamination with two mycotoxins (fumonisin B1 and fumonisin B2) produced by *Fusarium* mould, whereas the rest of the vegetables had no traces of mycotoxins (Table 5.2). Very low levels (0.001mg/kg) of fumonisin B1 and fumonisin B2 were detected in the vegetables. The presence of the mycotoxins might indicate that the vegetables were probably stored in conditions that favoured the growth of *Fusarium* mould. This finding is congruent with those of van der Walt and colleagues (2006), who noted that one of the vegetable samples (*Amaranthus hybridus* L*.*) traditionally dried and tested using similar approaches as those in our study contained fumonisin B1-3; meanwhile, some of the samples had low concentrations of fumonisin.

Table 5.2: Mycotoxins associated with traditional leafy vegetables sold within the Free State province

		Quantity detected (mg/kg)							
Sample name	**Place of collection**	**Aflatoxin B1**	**Deoxynevalenol**	**Nivalenol**	**Ochratoxin A**	**Zearalenol**	**Fumonisin B1**	**Fumonisin B2**	**Fumonisin B3**
Malva parviflora L. var. *parviflora*	Sasolburg	ND	ND	ND	ND	ND	ND	ND	ND
Lepidium capense L.	Dewetsdorp	ND	ND	ND	ND	ND	ND	ND	ND
Urtica dioica L.	Qwa-Qwa	ND	ND	ND	ND	ND	ND	ND	ND
Amaranthus hybridus L.	Qwa-Qwa	ND	ND	ND	ND	ND	*<LOQ	ND	ND
Chenopodium album L.	Thaba-Nchu	ND	ND	ND	ND	ND	ND	ND	ND
Chenopodium album L.	Qwa-Qwa	ND	ND	ND	ND	ND	*<LOQ	ND	ND
Chenopodium album L.	Bloemfontein	ND	ND	ND	ND	ND	0,001	0,001	ND
Cucurbita maxima	Bloemfontein	ND	ND	ND	ND	ND	ND	ND	ND
Rorippa nudiuscula Thell.	Bloemfontein	ND	ND	ND	ND	ND	ND	ND	ND
Amaranthus hybridus L.	Thaba-Nchu	ND	ND	ND	ND	ND	ND	ND	ND
Cucurbita maxima	Thaba-Nchu	ND	ND	ND	ND	ND	ND	ND	ND
Amaranthus hybridus L.	Bloemfontein	ND	ND	ND	ND	ND	ND	ND	ND
Lepidium capense L.	Thaba-Nchu	ND	ND	ND	ND	ND	ND	ND	ND

ND – Not Detected; LOQ (Limit of quantification) of all mycotoxins is 0.001 mg/kg

Even though low levels of these fumonisins were detected in the vegetables, they can still exert adverse effects on the health of individuals when consumed over an extended period of time (Ekwomadu, Akinola and Mwanza, 2021). For fungi to grow and produce mycotoxins in agricultural products, including vegetables and fruits, they have to be influenced by the following; the weather conditions before and after harvest, the crop variety, as well as the storage and processing conditions (Greco, Pardo and Pose, 2015; Koppen *et al*., 2010). For instance, *Fusarium* preferentially grows in humid conditions and at temperatures between 0-37°C and occurs typically in the fields (Thrane, 2004).

Exposure to fumonisins can be associated with carcinogenic effects, specifically, oesophagal cancer (Kamala *et al*., 2016; Ashiq, Hussain and Ahmad, 2014). In Tanzania, exposure to these toxins negatively impacts children's growth (Kamle *et al*., 2019). Fumonisin B1 is considered the most abundant and can inhibit embryonic sphingolipid synthesis, causing neural tube defects and blocking folate transport (Kamle *et al*., 2019; Waskiewicz *et al*., 2013).

Processing methods of TLVs may lead to increased fungal loads, however, there are preventative methods that could be put in place to reduce or eliminate fungi during postharvest processing. Such methods include; providing proper sanitation during processing, minimizing their water activity and enzyme functionality, and optimizing their temperature and relative humidity during storage (Kargwal *et al*., 2020).

On the other hand, the absence or very low levels of mycotoxins in the tested vegetables can have several positive implications. Firstly, this signifies a reduced health risk to the consumers. Mycotoxins can pose significant health hazards when consumed in large quantities, therefore, vegetables with minimal or no mycotoxins are safer for human consumption and can help prevent various health issues, including acute and chronic illnesses.

Secondly, the absence of mycotoxins indicates effective pre- and postharvest management practices: good agricultural practices, handling, and proper storage have been implemented to prevent fungal contamination and mycotoxin production. This reflects the diligence of farmers, distributors, and sellers in maintaining food safety standards. It also highlights the importance of food quality control.

Lastly, the low presence of mycotoxins can positively impact the economic aspect of traditional vegetables industry. Reducing mycotoxin contamination ensures that more vegetables meet the required safety standards, reducing the loss of products due to contamination and increasing sales. This not only safeguards the reputation of traditional vegetables producers but also contributes to food security and overall consumer confidence in the safety of the food supply chain. In general, the low levels of mycotoxins present in the tested vegetables is a win for both public health and the TLVs industry.

5.4 Conclusion

Based on the mycotoxin analysis conducted in this study, the results indicated that most traditional leafy vegetables sold in the local markets of the Free State province were free of mycotoxin contamination, whereas two vegetables (*Amaranthus hybridus* L. and *Chenopodium album* L.) had traces of mycotoxins, although within the acceptable limits for human consumption. The presence of low levels of mycotoxins in TLVs paves way for popularisation of the vegetables and proves that indigenous systems employed in processing TLVs are safe with regards to mycotoxins.

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CHAPTER 6

GENERAL DISCUSSION, CONCLUSIONS, AND RECOMMENDATIONS

6.1 General discussion

Traditional leafy vegetables (TLVs) are recognised across sub-Saharan Africa because of their nutritional value and health benefits (Yang and Keding, 2009; Vorster, van Zyl and Venter, 2007). Traditional leafy vegetables are enriched with nutrient and mineral content comprising of carbohydrates, proteins, vitamins and dietary fibre; however, they are regarded as poor people's food and knowledge relating to these said plants is termed "backward", thus the reluctance to transfer knowledge linking their habitat and importance to the younger people who are now faced with changing social values and migration from rural areas where the TLVs are being cultivated to cities wherein they are ignored and neglected for the use of exotic plants (van der Walt *et al*., 2009). Although overlooked by most of the population, TLVs can create exceptional changes to diversify farming systems, ensure food security, alleviate poverty, improve human health, and increase income (Njeme, Goduka and George, 2014). Therefore, TLVs should be collected, later preserved, and documented so that the knowledge will serve valuable purposes in crop improvement and maintenance of local cultures and traditions.

The current study investigated whether the traditional vegetables were still in use in the province and if they were safe for human consumption. The overall objective or aim was to assess the mycotoxigenic fungi and mycotoxin contamination of TLVs obtained from Free State, South Africa. To fulfil the purpose of the study, several specific objectives were targeted, amongst which included conducting a market survey in the Free State province to determine the most commonly sold TLVs; to identify the preservation methods, storage conditions and packaging of TLVs, and lastly, to identify fungi and the mycotoxins present in TLVs.

Accordingly, a market survey was conducted through purposive and random sampling, using questionnaires drafted in English and translated during data collection since most respondents had limited education. The majority of respondents were men. The findings through the market survey (Chapter 3) provided evidence that traditional leafy vegetables are still being utilised, either as food or medicine. In addition, the study showed that consumers of TLVs are more common in rural than urban populations, primarily because in rural communities, TLVs are more easily accessed or harvested during the growing season. This is affirmed by Lewu and Mavengahama (2010), who noted that most provinces in South Africa are still facing the challenge of high levels of poverty, notably among the rural communities, with the poverty level cited as high as 78.2 %, compelling the indigenes to resort to nature for subsistence. Nevertheless, TLVs commonly consumed by the population in the Free State were documented.

Ten different plant samples were collected and identified from the vendors. The ten traditional leafy vegetables belonged to different plant families; Asteraceae, Urticaceae, Brassicaceae, Amaranthaceae, Campanulaceae, Chenopodiaceae, and Malvaceae. More than thirty percent (33.33%) belonged to the Brassicaceae family, whereas 11.11% constituted the remaining families. Most of the TLVs were sold as dry for medicinal purposes, which can be explained by the fact that traditional medicine remains the first point of call for the majority of the inhabitants in search of primary healthcare needs (Aremu & Pendota, 2021). Dried vegetables could be stored for months, enabling the vendors to generate income even in dry seasons. The least-dried vegetables were amaranth and pumpkin, as most consumers preferred them fresh. They were mainly prepared as a relish for maize porridge, and the preparation methods differed among the various ethnic groups. Creating awareness of the importance of TLVs and their marketing potential could improve their utilisation by the youth, the middle-aged, and the old.

The dried aerial parts of five plants, including *Cucurbita maxima* (mokopu), *Chenopodium album* L. (seruoe), *Rorippa nudiuscula* Thell. (papasane), *Amaranthus hydridus* L. (Theepe) and *Lepidium capense* Thunb. (Qhela) that were stored in either newspapers, polyethylene or polypropylene bags were evaluated for mycotoxigenic fungal and mycotoxins contamination. The plants were powdered while observing aseptic conditions to prevent contamination of the powdered plants. Samples of each powdered plant were serially diluted and cultivated on malt extract agar and potato dextrose agar to enumerate the fungal counts that showed the level of contamination of the plants. The fungal counts occurred in the range 1-109_cfu/g and appeared as white, greyish, greenish-black and black mycelium. According to Klapeć *et al*. (2021), these contaminants pose health threats to certain categories of individuals consuming fresh vegetables, mainly the immunocompromised, the atopicas, the elderly and the children susceptible to food allergy following ingestion of moulds. The findings indicated that fungal contamination of the TLVs is possible and could have occurred through the preparation, preservation, and storage of the vegetables (Mulaosmanovic *et al*., 2021).

Pure fungal cultures were employed for DNA extraction and amplification to identify the fungal contaminants. The extracted DNA from the pure fungal cultures were amplified using specific primers, and the amplicons were purified and sequenced. The sequences were subjected to a BLASTn search compared with already identified sequences located in the NCBI database to confirm the identity of the contaminants. Our findings revealed that the fungal contaminants were *Epicoccum sorghinum, Alternaria alternata, Phoma sp, Didymella sp., Cladosporium sp, Rhizopus oryzae, Didymella macrostoma, and Didymella glomerata*. *Epicoccum sorghinum* and *Rhizopus oryzae* were the most prominent fungi (33.33%), followed by *Phoma sp*. (22.22%), and only *Fusarium, Alternaria alternata* and *Epicoccum sorghinum* are known to produce mycotoxins.

Following the identification of fungal species in TLVs, we determined the presence of eight (8) mycotoxins; aflatoxin B1, deoxynivalenol, nivalenol, ochratoxin A, zearalenone, fumonisin B1, fumonisin B2 and fumonisin B3. A rapid and sensitive ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-ESI-MS/MS) technology was used to analyse and quantify the presence of the eight (8) mycotoxins TLVs. Our data revealed the presence of Fumonisin B1 and Fumonisin B2 produced by *Fusarium* in *Chenopodium album* L. and *Amaranthus hybridus* L., showing contamination at meagre quantities. It can be concluded that TLVs are safe for human consumption as very little to no mycotoxins were detected in the vegetables. This proves that traditional systems for food processing and handling are safe to use as they do not allow food contamination.

6.2 Conclusions

Following the interpretation of our data, the following conclusions are worth mentioning.

1) Traditional leafy vegetables (TLVs) are mainly sold to be used as food (relish) or medicine.
2) TLVs are seasonal and highly perishable; hence different methods are used to preserve them when they are out of season. More dried than fresh TLVs were sold in informal markets.
3) Most TLVs are collected from farm fields, gardens, and the veld. Very few of them, such as *amaranth*, are cultivated.
4) TLVs can be stored in plastic bags, newspapers, and empty maize meal bags.
5) Only one method is used to preserve TLVs, which is sun drying. They are dried for a few days or until completely dried.
6) TLVs are dried on flat surfaces in the open, exposing them to contaminants such as dust and insects.
7) Dried TLVs were contaminated with fungi. Approximately 205 fungal colonies were counted from the vegetable samples, and the sample with the highest count was *Cucurbita maxima*.
8) Nine fungal species were isolated from the vegetable samples, including *Epicoccum sorghinum*, *Alternaria alternata*, *Cladosporium sp.*, *Rhizopus oryzae*, *Nothophoma quercina*, *Fusarium sp.*, *Didymella microstoma*, *Didymella glomerata* and *Phoma sp.*
9) Two vegetable samples (*Chenopodium album* L. and *Amaranthus hybridus* L.) were contaminated with Fumonisin B1 and Fumonisin B2 produced by *Fusarium sp*. although mycotoxin quantities were low.

6.3 Recommendations

The following recommendations are made based on the observations during the study:

1) To promote knowledge of TLVs and their use among the youth, the integration TLVs into the formal markets is encouraged. This may also be augmented by updating preparation and preservation methods.
2) Quality assurance issues and adopting Hazard Analysis Critical Control Point (HACCP) Principles must be addressed when developing traditional plant products.
3) Improved preservation and storage methods (e.g., airtight containers) would help increase the shelf life of dried TLVs and minimise contamination. Preservation of TLVs by appropriate technologies could improve utilisation and provide an opportunity to increase sales.
4) Future studies could be extended to other provinces to identify the availability of TLVs, preservation methods, storage practices, and consumer safety and hygiene standards.
5) Based on the research findings from this study, further studies are required to isolate and identify mycotoxigenic fungi and mycotoxins in TLVs. This will, in turn, help in preventing and controlling contamination.

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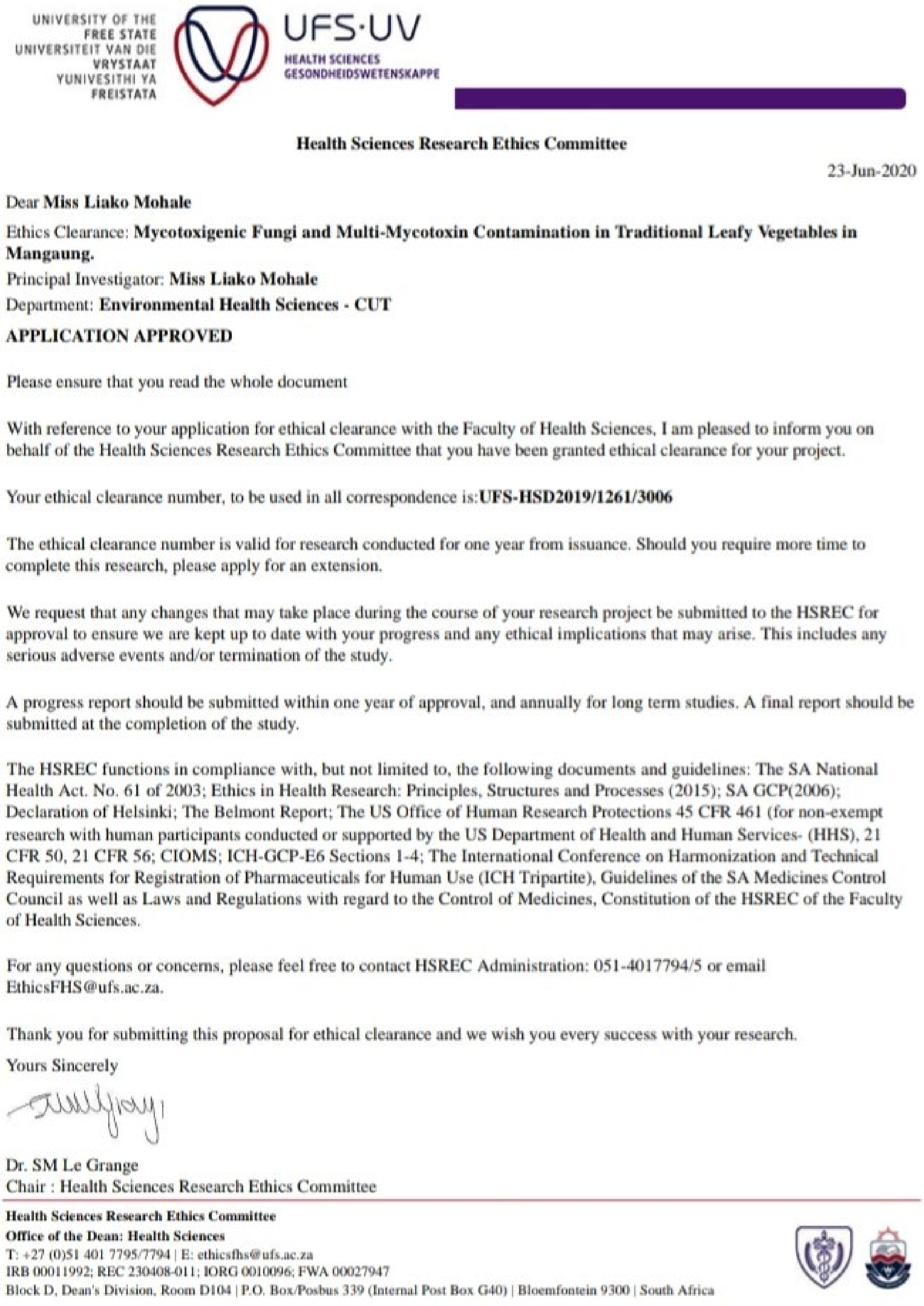
Annexures

Annexure A: Ethical Clearance



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

UFS·UV
HEALTH SCIENCES
GESONDHEIDSWETENSKAPPE

Health Sciences Research Ethics Committee

23-Jun-2020

Dear **Miss Liako Mohale**

Ethics Clearance: **Mycotoxigenic Fungi and Multi-Mycotoxin Contamination in Traditional Leafy Vegetables in Mangaung.**
Principal Investigator: **Miss Liako Mohale**
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-HSD2019/1261/3006**

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.

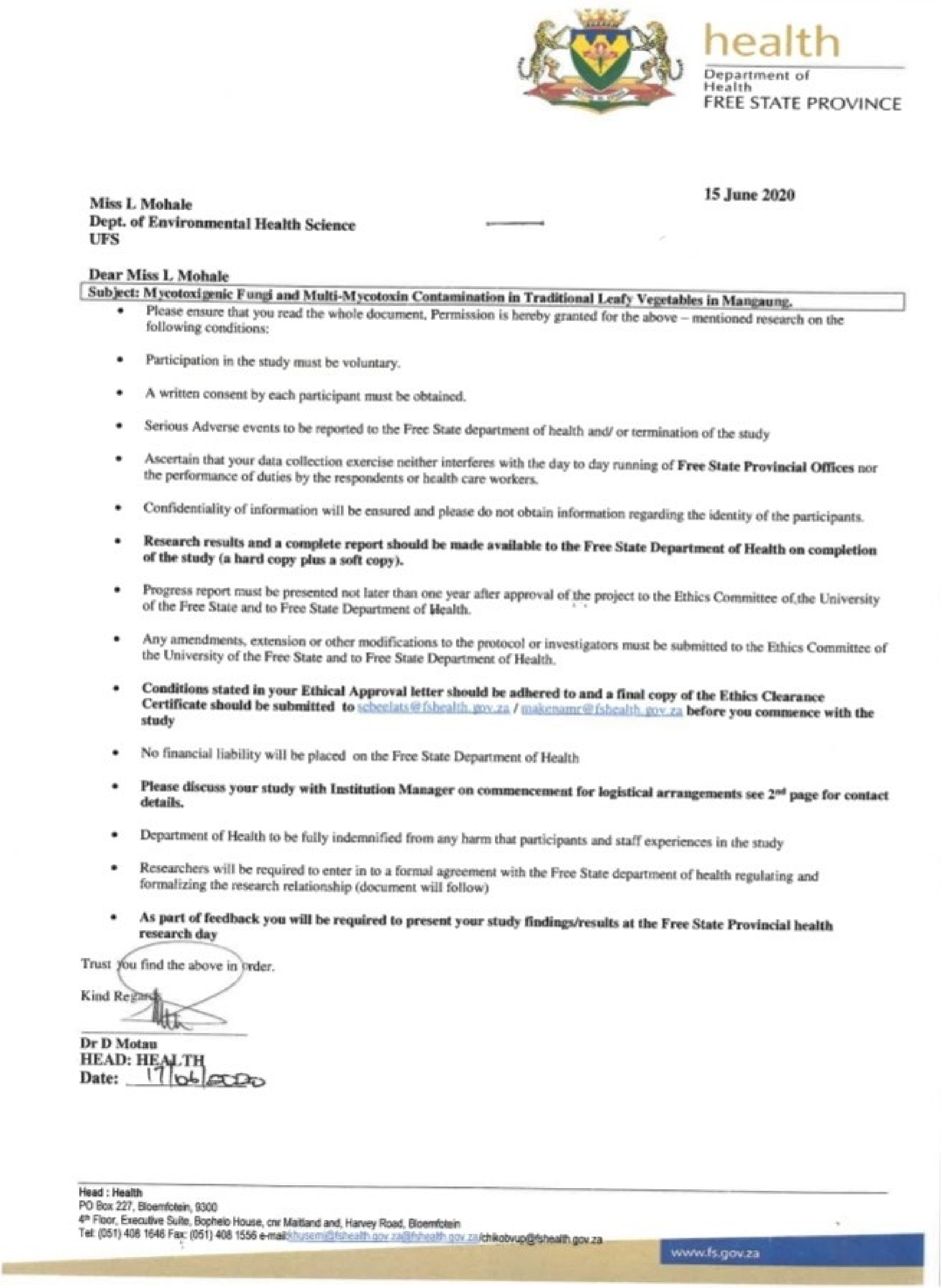
Thank you for submitting this proposal for ethical clearance and we wish you every success with your research.

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
Block D, Dean's Division, Room D104 | P.O. Box/Posbus 339 (Internal Post Box G40) | Bloemfontein 9300 | South Africa

Annexure B: Letter from the Department of Health



health
Department of
Health
FREE STATE PROVINCE

15 June 2020

Miss L Mohale
Dept. of Environmental Health Science
UFS

Dear Miss L Mohale

Subject: Mycotoxigenic Fungi and Multi-Mycotoxin Contamination in Traditional Leafy Vegetables in Mangaung.

- Please ensure that you read the whole document, Permission is hereby granted for the above – mentioned research on the following conditions:
- Participation in the study must be voluntary.
- A written consent by each participant must be obtained.
- Serious Adverse events to be reported to the Free State department of health and/ or termination of the study
- Ascertain that your data collection exercise neither interferes with the day to day running of **Free State Provincial Offices** nor the performance of duties by the respondents or health care workers.
- Confidentiality of information will be ensured and please do not obtain information regarding the identity of the participants.
- **Research results and a complete report should be made available to the Free State Department of Health on completion of the study (a hard copy plus a soft copy).**
- Progress report must be presented not later than one year after approval of the project to the Ethics Committee of the University of the Free State and to Free State Department of Health.
- Any amendments, extension or other modifications to the protocol or investigators must be submitted to the Ethics Committee of the University of the Free State and to Free State Department of Health.
- **Conditions stated in your Ethical Approval letter should be adhered to and a final copy of the Ethics Clearance Certificate should be submitted to** scbeelats@fshealth.gov.za / makenamr@fshealth.gov.za **before you commence with the study**
- No financial liability will be placed on the Free State Department of Health
- **Please discuss your study with Institution Manager on commencement for logistical arrangements see 2nd page for contact details.**
- Department of Health to be fully indemnified from any harm that participants and staff experiences in the study
- Researchers will be required to enter in to a formal agreement with the Free State department of health regulating and formalizing the research relationship (document will follow)
- **As part of feedback you will be required to present your study findings/results at the Free State Provincial health research day**

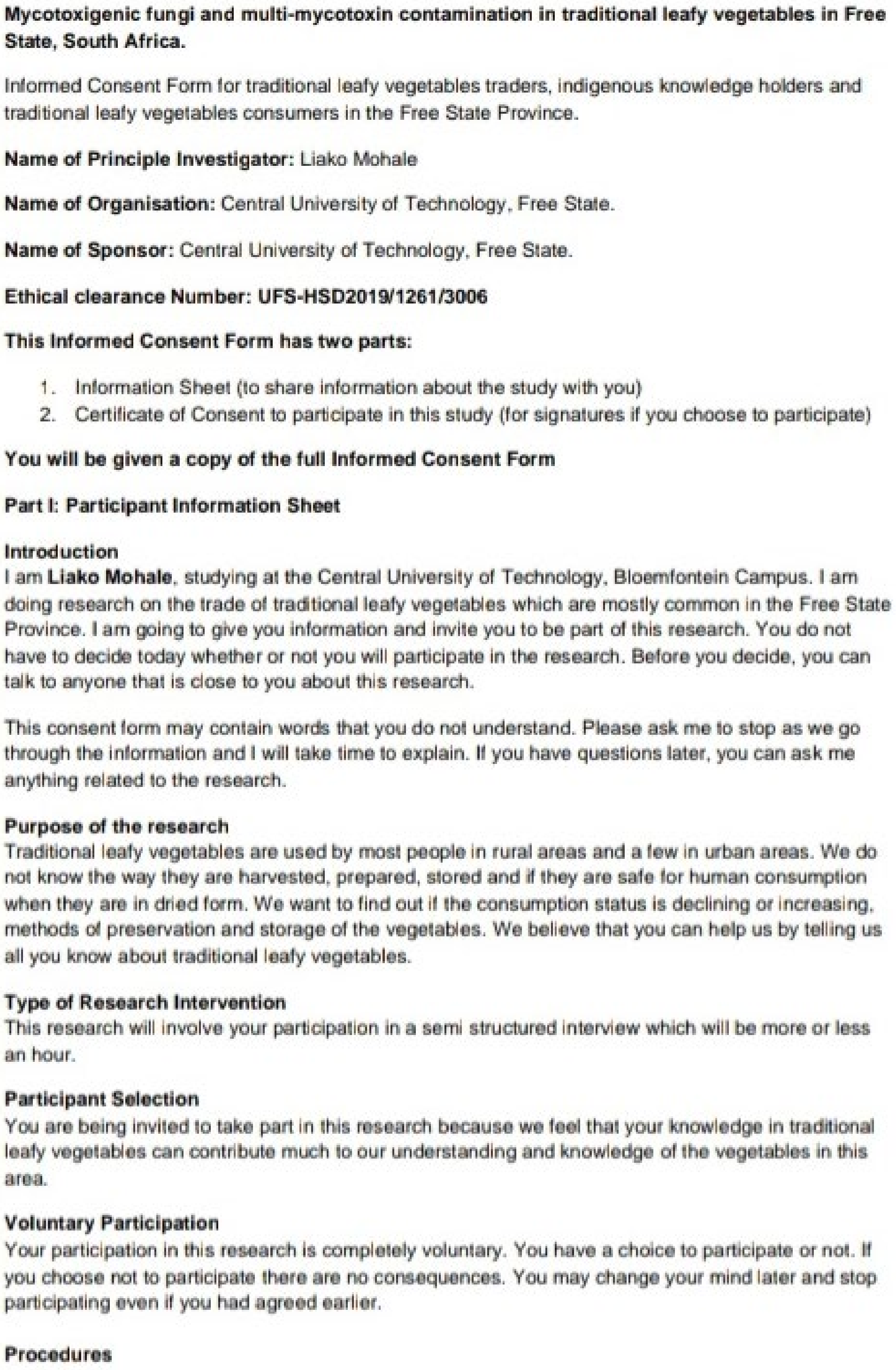
Trust you find the above in order.

Kind Regards

Dr D Motau
HEAD: HEALTH
Date: 17/06/2020

Head : Health
PO Box 227, Bloemfontein, 9300
4th Floor, Executive Suite, Bophelo House, cnr Maitland and, Harvey Road, Bloemfontein
Tel: (051) 408 1646 Fax: (051) 408 1556 e-mail:khusemj@fshealth.gov.za@fshealth.gov.za/chikobvup@fshealth.gov.za
www.fs.gov.za

Annexure C: Consent Form



Mycotoxigenic fungi and multi-mycotoxin contamination in traditional leafy vegetables in Free State, South Africa.

Informed Consent Form for traditional leafy vegetables traders, indigenous knowledge holders and traditional leafy vegetables consumers in the Free State Province.

Name of Principle Investigator: Liako Mohale

Name of Organisation: Central University of Technology, Free State.

Name of Sponsor: Central University of Technology, Free State.

Ethical clearance Number: UFS-HSD2019/1261/3006

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 **Liako Mohale**, studying at the Central University of Technology, Bloemfontein Campus. I am doing research on the trade of traditional leafy vegetables which are mostly common in the Free State Province. I am going to give you information and invite you to be part of 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 that is close to you about this 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 anything related to the research.

Purpose of the research

Traditional leafy vegetables are used by most people in rural areas and a few in urban areas. We do not know the way they are harvested, prepared, stored and if they are safe for human consumption when they are in dried form. We want to find out if the consumption status is declining or increasing, methods of preservation and storage of the vegetables. We believe that you can help us by telling us all you know about traditional leafy vegetables.

Type of Research Intervention

This research will involve your participation in a semi structured interview which will be more or less an hour.

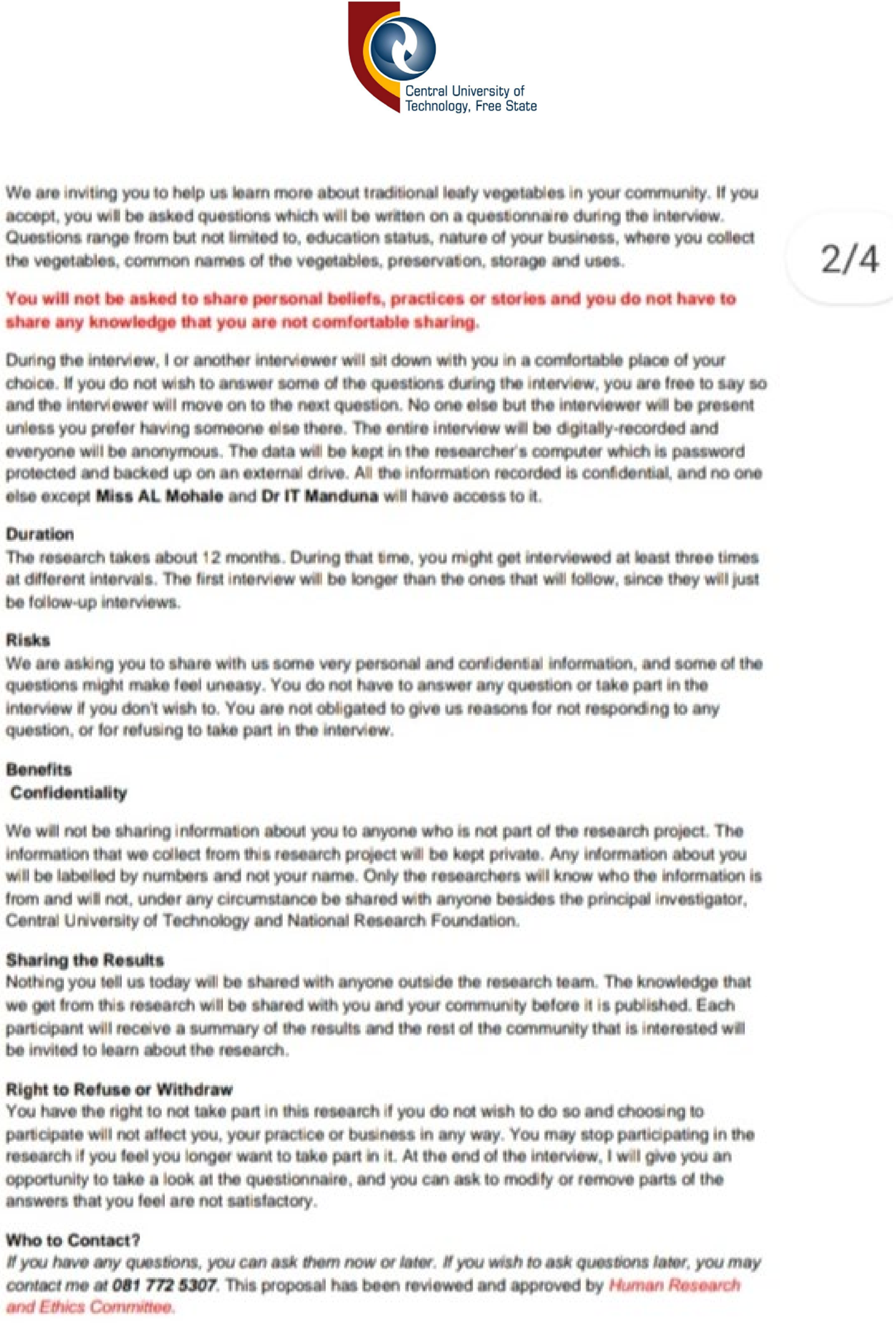
Participant Selection

You are being invited to take part in this research because we feel that your knowledge in traditional leafy vegetables can contribute much to our understanding and knowledge of the vegetables in this area.

Voluntary Participation

Your participation in this research is completely voluntary. You have a choice 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 had agreed earlier.

Procedures



Central University of Technology, Free State

2/4

We are inviting you to help us learn more about traditional leafy vegetables in your community. If you accept, you will be asked questions which will be written on a questionnaire during the interview. Questions range from but not limited to, education status, nature of your business, where you collect the vegetables, common names of the vegetables, preservation, storage 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 place of your choice. If you do not wish to answer some of the questions during the interview, you are free to say so and the interviewer will move on to the next question. No one else but the interviewer will be present unless you prefer having someone else there. The entire interview will be digitally-recorded and everyone will be anonymous. The data will be kept in the researcher's computer which is password protected and backed up on an external drive. All the information recorded is confidential, and no one else except **Miss AL Mohale** and **Dr IT Manduna** will have access to it.

Duration

The research takes about 12 months. During that time, you might get interviewed at least three times at different intervals. The first interview will be longer than the ones that will follow, since they will just be follow-up interviews.

Risks

We are asking you to share with us some very personal and confidential information, and some of the questions might make feel uneasy. You do not have to answer any question or take part in the interview if you don't wish to. You are not obligated to give us reasons for not responding to any question, or for refusing to take part in the interview.

Benefits

Confidentiality

We will not be sharing information about you to anyone who is not part of the research project. The information that we collect from this research project will be kept private. Any information about you will be labelled by numbers and not your name. Only the researchers will know who the information is from and will not, under any circumstance be shared with anyone besides the principal investigator, Central University of Technology and National Research Foundation.

Sharing the Results

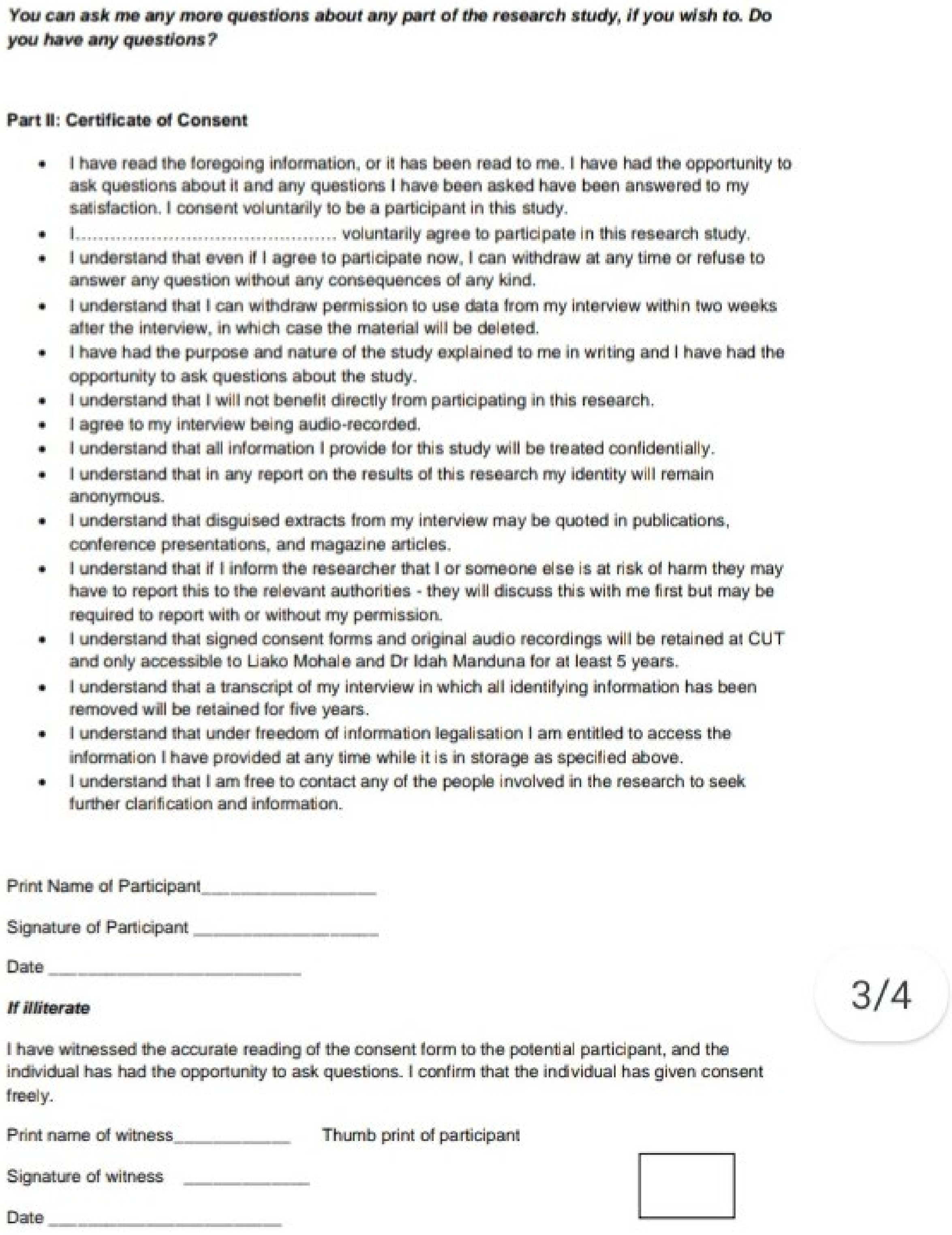
Nothing you tell us today will be shared with anyone outside the research team. The knowledge that we get from this research will be shared with you and your community before it is published. Each participant will receive a summary of the results and the rest of the community that is interested will be invited to learn about the research.

Right to Refuse or Withdraw

You have the right to not take part in this research if you do not wish to do so and choosing to participate will not affect you, your practice or business in any way. You may stop participating in the research if you feel you longer want to take part in it. At the end of the interview, I will give you an opportunity to take a look at the questionnaire, and you can ask to modify or remove parts of the answers that you feel are not satisfactory.

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 me at **081 772 5307**.* 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, and magazine articles.
- I understand that if I inform the researcher that I 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 Liako Mohale and Dr Idah 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 __________________________

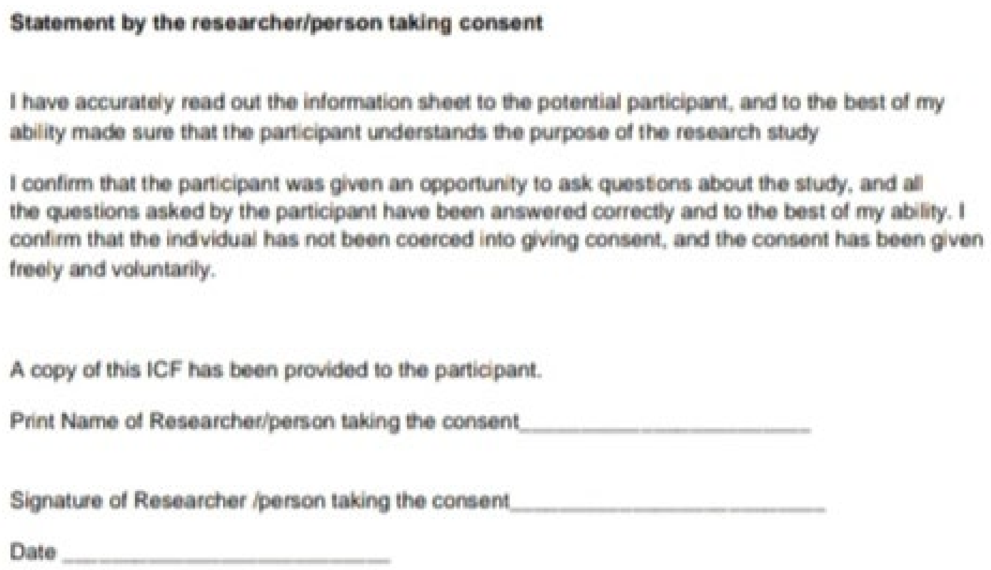
If illiterate

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.

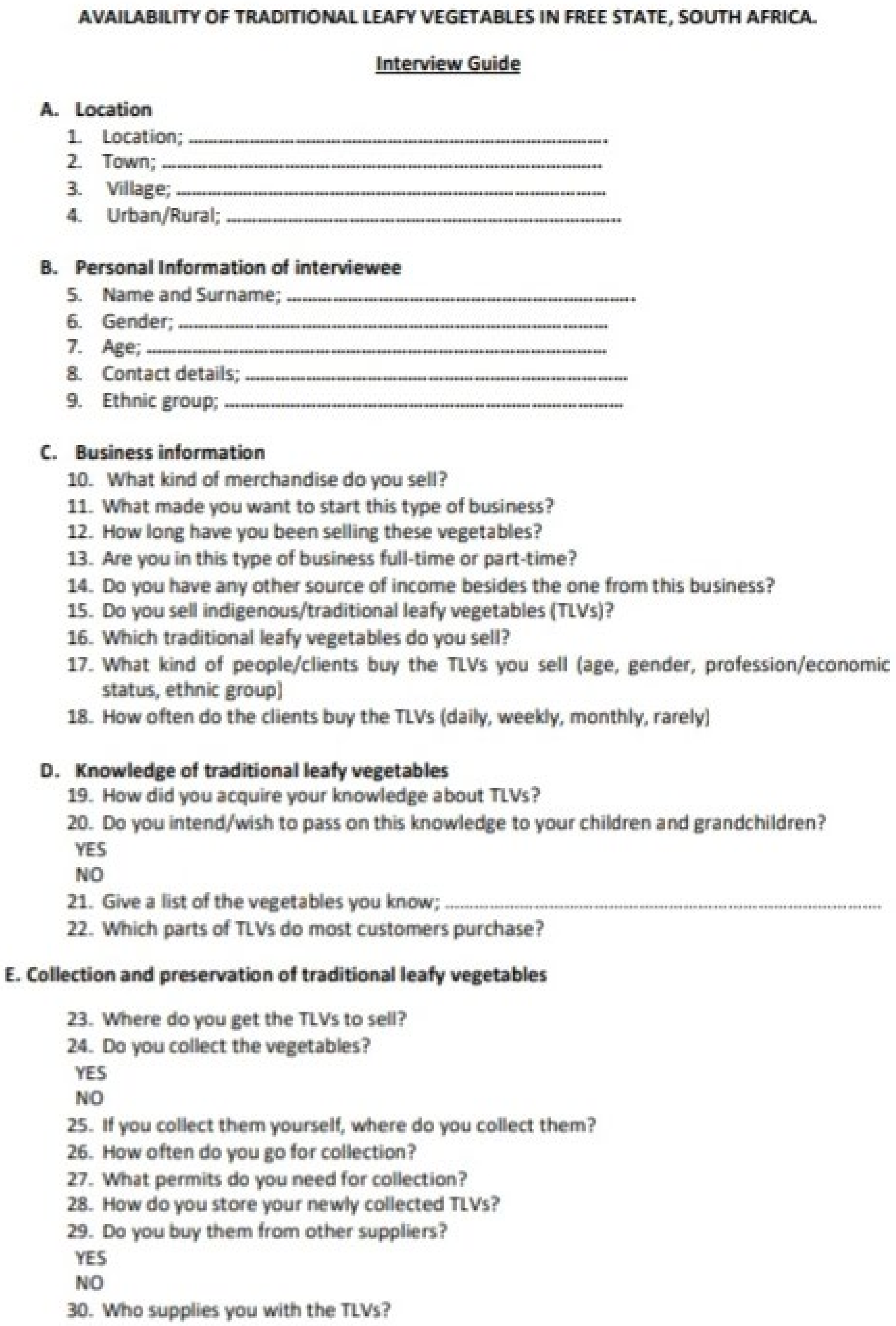
A copy of this ICF has been provided to the participant.

Print Name of Researcher/person taking the consent______________________

Signature of Researcher /person taking the consent________________________

Date ________________________

Annexure D: Interview Guide



AVAILABILITY OF TRADITIONAL LEAFY VEGETABLES IN FREE STATE, SOUTH AFRICA.

Interview Guide

A. Location

1. Location; ……………………………………………………………………………
2. Town; ……………………………………………………………………………
3. Village; ……………………………………………………………………………
4. Urban/Rural; ……………………………………………………………………………

B. Personal Information of interviewee

5. Name and Surname; ……………………………………………………………………
6. Gender; ……………………………………………………………………………
7. Age; ……………………………………………………………………………
8. Contact details; ……………………………………………………………………
9. Ethnic group; ……………………………………………………………………

C. Business information

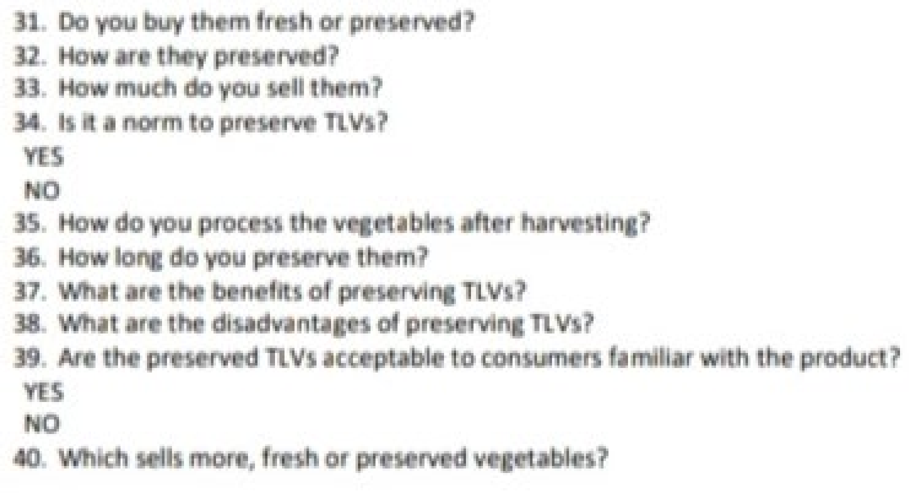
10. What kind of merchandise do you sell?
11. What made you want to start this type of business?
12. How long have you been selling these vegetables?
13. Are you in this type of business full-time or part-time?
14. Do you have any other source of income besides the one from this business?
15. Do you sell indigenous/traditional leafy vegetables (TLVs)?
16. Which traditional leafy vegetables do you sell?
17. What kind of people/clients buy the TLVs you sell (age, gender, profession/economic status, ethnic group)
18. How often do the clients buy the TLVs (daily, weekly, monthly, rarely)

D. Knowledge of traditional leafy vegetables

19. How did you acquire your knowledge about TLVs?
20. Do you intend/wish to pass on this knowledge to your children and grandchildren?
    YES
    NO
21. Give a list of the vegetables you know; ……………………………………………………………………
22. Which parts of TLVs do most customers purchase?

E. Collection and preservation of traditional leafy vegetables

23. Where do you get the TLVs to sell?
24. Do you collect the vegetables?
    YES
    NO
25. If you collect them yourself, where do you collect them?
26. How often do you go for collection?
27. What permits do you need for collection?
28. How do you store your newly collected TLVs?
29. Do you buy them from other suppliers?
    YES
    NO
30. Who supplies you with the TLVs?



31. Do you buy them fresh or preserved?
32. How are they preserved?
33. How much do you sell them?
34. Is it a norm to preserve TLVs?

YES
NO

35. How do you process the vegetables after harvesting?
36. How long do you preserve them?
37. What are the benefits of preserving TLVs?
38. What are the disadvantages of preserving TLVs?
39. Are the preserved TLVs acceptable to consumers familiar with the product?

YES
NO

40. Which sells more, fresh or preserved vegetables?