

**FUNGAL AND AFLATOXIN CONTAMINATION OF HERBAL
MEDICINES SOLD IN LIRA AND GULU CITIES, NORTHERN UGANDA**

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Declaration and Approval

I, Orech Walter, do hereby declare that this work is original and is a result of my effort and it has never been submitted for any academic award in this or any academic institution.

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Approval

This research dissertation entitled '**Fungal and aflatoxin contamination of herbal medicine sold in Lira and Gulu cities, northern Uganda**' was submitted to the Institute of Research and Graduate Training with the approval of the undersigned supervisors.

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Dedication

This work is dedicated to my parents, friends, and colleagues for their unwavering support rendered to me throughout this academic journey. The kind of support you offered me has been immense and memorable.

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List of abbreviations

Abbreviations	Word meanings
µg/kg	Microgram per kilogram
AF	Aflatoxin
AFB ₁	Aflatoxin B ₁
AFB ₂	Aflatoxin B ₂
AFG ₁	Aflatoxin G ₁
AFG ₂	Aflatoxin G ₂
AFM ₁	Aflatoxin M ₁
AFM ₂	Aflatoxin M ₂
BLAST	Basic Local Alignment Search Tool

CTAB	Cetyltrimethylammonium Bromide
DNA	Deoxyribonucleic Acid
EAGC	Eastern Africa Grain Council
EDI	Estimated Daily Intake
ELISA	Enzyme-Linked Immunosorbent Assay
EU	European Union
GUREC	Gulu University Ethical Research Committee
HCC	Hepatocellular Carcinoma
HI	Hazard Index
HMP	Herbal Medicinal Product
IARC	International Agency for Research on Cancer
ITS	Internal Transcribed Spacer
MEGA	Molecular Evolutionary Genetics Analysis
NARO	National Agricultural Research Organization
NCBI	National Center for Biotechnology Information
NDA	National Drug Authority
ng/g	Nanogram per gram
PCR	Polymerase Chain Reaction
PDA	Potato Dextrose Agar
SPSS	Statistical Package for the Social Sciences
TD50	Toxic Dose 50
UNBS	Uganda National Bureau of Standards
UV light	Ultra violet light
WHO	World Health Organisation

Abstract

In middle and low income countries, the use of herbal medicinal products has significantly increased. Therefore, ensuring the safety of herbal products from fungal contamination is a public health concern. This cross-sectional study aimed to assess aflatoxin and fungal contamination in herbal medicines sold in Gulu and Lira cities, northern Uganda. A total of 52 herbal medicine samples were collected. Moisture content was determined by oven-drying method, aflatoxin extraction was done using 70% methanol, and analysis was conducted using ELISA. Fungal identification was performed through DNA extraction, amplification, and sequencing. Statistical analysis was conducted using SPSS version 25. Out of the 52

samples analysed, aflatoxin and fungal incidences were 35% and 34.6%, respectively. While most aflatoxin contamination levels were below regulatory limits, 11.1% exceeded the European Pharmacopoeia permissible level of 5 µg/kg. The study also revealed a positive correlation between moisture content and aflatoxin contamination. To mitigate aflatoxin contamination and ensure the safety of herbal medicines, it is imperative to implement improved processing techniques, packaging, storage, and quality control measures. Common fungal species identified included *Aspergillus flavus*, *Aspergillus fumigatus*, and *Pichia kudriavzevii*. *Pichia kudriavzevii* was identified as the most prevalent fungi. The estimated daily intake and hazard index values indicated a low health risk for consumers. Although the short-term health risk appears low, long-term consumption may lead to adverse health effects. This study highlights the urgent need for increased awareness and action to safeguard public health in relation to herbal medicine consumption in northern Uganda.

CHAPTER ONE: INTRODUCTION

1.1 Background

Aflatoxins are naturally occurring mycotoxins produced by certain fungi like *Aspergillus parasiticus* and *A. flavus* (Liew & Mohd-Redzwan, 2018; Syamilah *et al.*, 2022). Aflatoxin is a type of secondary fungal metabolite that poses a threat to humans and animals (Taniwaki & Pitt, 2019). Globally, there are over 300 mycotoxins, with aflatoxins, fumonisins, ochratoxins, trichothecenes, and zearalenone being the most significant (El-Sayed *et al.*, 2022). Repeated dietary exposures to aflatoxins have been found to have significant health consequences for both humans and animals (Sarma *et al.*, 2017). Aflatoxin may exhibit carcinogenic, neurotoxic, mutagenic, hepatotoxic, teratogenic, estrogenic, cytotoxic, and nephrotoxic properties, and can occasionally induce immunosuppression in humans (Awuchi *et al.*, 2021). Furthermore, aflatoxin B₁, classified as a Group I carcinogen, significantly increases the risk of hepatocellular carcinoma (HCC) (Gong *et al.*, 2016). Aflatoxin B₁ is the most potent liver carcinogenic agent and is found in greater concentrations than any other occurring aflatoxins (Hamid *et al.*, 2013).

The widespread utilisation of medicinal herbs globally has raised significant safety concerns regarding contamination by microbial organisms (Rizzo *et al.*, 2014). The majority of fungi are toxigenic, whereas certain non-toxigenic species may contribute a mouldy odour and taste (Sekar *et al.*, 2012). In Argentina, Rizzo *et al.* (2004) reported that 52% of 152 dried medicinal herbs were contaminated with *Aspergillus* species, whereas 16% exhibited contamination by *Fusarium* species. In Brazil, Bugno *et al.* (2006) found that 89.9% of the isolates from 91 samples of medicinal plants were *Aspergillus* and *Penicillium*. A survey conducted in India reported that 47% of the medicinal herb and spice samples exceeded the acceptable fungal load. Aflatoxin-producing fungi can contaminate crops in the field, at the time of harvest, and during storage (Kumar *et al.*, 2021). In the pre-harvest stage, medicinal herbs are susceptible to indigenous fungi in the soil where they were cultivated. The dried part of medicinal herbs may be susceptible to fungal contamination during post-harvest processing. Aflatoxins are a consequence of fungal growth and, therefore, also an indicator of hygienic deficits during production and storage of herbal medicine (Altyn & Twarużek, 2020). The quality and safety of herbal products are closely related to compliance with the principles of good agricultural practice at every stage of production (WHO, 2003). Contamination occurs when safety practices are not followed during the production of herbal

medicine. Mycological and aflatoxin contamination of traditional herbal medicines is common (Katerere *et al.*, 2008). In a study conducted by Zhang *et al.* (2019), it was observed that 28 out of 32 samples obtained from China were found to be contaminated with aflatoxin. Furthermore, the contamination was present throughout all stages of processing. Ahmad *et al.* (2014) examined 30 herbal samples from Pakistan and detected aflatoxin and ochratoxin in 30% and 26.70% of the samples, respectively. Another study by Ariño *et al.* (2007) revealed that all 30 samples of licorice and its derivative products collected from Spain were contaminated with ochratoxin. Aflatoxin contamination of herbal medicine have been reported for example research on commercial herbal formulations in Malaysia and Indonesia found aflatoxin B₁ (AFB₁), AFB₂, AFG₁ and AFG₂ contamination levels averaging 0.26 µg/kg, 0.07 µg/kg, 0.10 µg/kg and 0.03 µg/kg, respectively. Estimated daily intake was approximately 0.022 ng/kg body weight (Ali *et al.*, 2005). A separate Thai study detected aflatoxin contamination in 18% of 28 herbal medicine products, with levels ranging from 1.7 to 14.3 ng/g (Tassaneeyakul *et al.*, 2004).

The mycotoxins of concern in Sub-Saharan Africa are aflatoxins, fumonisins, ochratoxins, trichothecenes, and zearalenone (Wu *et al.*, 2010). Aflatoxin alone is responsible for about 250,000 hepatocellular carcinoma-related deaths per year in sub-Saharan Africa, making aflatoxin contamination in food a serious public health concern (Wagacha & Muthomi, 2008). A study done in Nigeria reported that aflatoxin B₁ was the most commonly recorded aflatoxin in the samples that was above the acceptable levels outlined by the World Health Organization (Ezekwesili-Ofili *et al.*, 2014). Even in small quantities, aflatoxin exposure has been reported to cause acute mycotoxicosis, a risk factor for human diseases like cancer and stunting in children (Matumba *et al.*, 2014). Acute liver necrosis and mortality are usually the results of prolonged exposure to aflatoxins at a dose above safe limits (4–30 ng/L) (Kaaya & Kyamuhangire, 2006). Therefore, it is assumed that some foods consumed could be the source of the carcinogens associated with cancer and other diseases (Kitya *et al.*, 2010).

In Uganda, the predominant mycotoxins are aflatoxins and fumonisins, with aflatoxins being the most prevalent. The aflatoxins of importance in Uganda are Aflatoxin B₁ (AFB₁), Aflatoxin B₂ (AFB₂), Aflatoxin G₁ (AFG₁), and Aflatoxin G₂ (AFG₂) (Kitya *et al.*, 2010). Cases of aflatoxin mortality have been reported in Uganda as a result of the consumption of food significantly contaminated with aflatoxin, (Asiki *et al.*, 2014). In addition, the risk of hepatoma has escalated in Uganda due to the high prevalence of hepatitis B and frequent exposure to aflatoxins.

Furthermore, babies born to mothers exposed to high levels of aflatoxins frequently had lower birth weights (Serck-Hanssen, 1970). Growth retardation, lowered immunity, and protein deficiency are manifestations of infant exposure to elevated aflatoxin levels, whether through breast milk or supplementary feeding (Lauer *et al.*, 2019). Thus, exposure to aflatoxin is believed to contribute to the high stunting prevalence of 35–40% among infants in Uganda (Asiki *et al.*, 2014), with more than 90% of the population impacted by high aflatoxin exposure, as evidenced by its presence in their body fluids (Kang *et al.*, 2015). In Uganda, there is substantial consumption of herbal medicines due to cultural, social, and economic factors as well as the availability of a vast biodiversity of flora used to make herbal products (Ssenku *et al.*, 2022). Nonetheless, the contamination of herbal medicines with aflatoxin-producing fungi has been commonly reported in several studies conducted elsewhere (Katerere *et al.*, 2008). Currently, there is inadequate data on the aflatoxin contamination and safety of herbal medicines in Uganda. Therefore, the present study aims to examine the level of aflatoxin, associated health risks and fungal contamination in herbal medicines sold in northern Uganda in order to raise public awareness of aflatoxin.

1.2 Statement of the problem

Poor storage and processing practices put the quality and safety of locally produced herbal medicine at risk (de Sousa Lima *et al.*, 2020). The most significant threat to the safety of herbal medicines is contamination by various microorganisms that may adhere to the leaves, stems, flowers, seeds, and roots used to make herbal medicines (de Sousa Lima *et al.*, 2020). Alternately, microbes can cause contamination during harvesting, handling, open-air drying, preservation, and storage. As a result, the locally made herbal medications are particularly susceptible to contamination by aflatoxigenic fungi such as *Aspergillus spp.*, which produces aflatoxin (Kosalec *et al.*, 2009).

Aflatoxin contamination poses a significant public health threat to consumers of herbal medicines sold in northern Uganda. Despite the wide spread use of herbal medicines among the population, there is lack of scientific data on the extent of aflatoxin contamination in the region. Studies conducted elsewhere in Uganda have revealed alarming levels of aflatoxin in herbal products associated with increased risk of liver cancer, immune suppression, and other health issues. A study conducted in Kampala, Uganda, revealed that 60% of herbal medicines were contaminated with aflatoxin, with an estimated daily intake ranging from 5.61×10^{-2} to 1.51×10^{-1} $\mu\text{g/kg/bw/day}$ for both Ugandan children and adults (Onen *et al.*, 2021).

The International Agency for Research on Cancer (IARC) monograph classifies aflatoxin as a Group 1 carcinogen (IARC, 2012). Regrettably, consuming herbal products contaminated with aflatoxins at sub-lethal doses results in long-term carcinogenic effects. According to Liu & Wu *et al.* (2010), about 40% of liver cancer incidences in Africa have been attributed to dietary aflatoxin exposure. Besides the carcinogenic effects, aflatoxin is known to be cytotoxic, nephrotoxic, mutagenic, and teratogenic (Ali *et al.*, 2005). Similarly, in Tororo (Uganda), the shoot powder of *Phyllanthus amarus*, used as a raw material for making herbal medicine, was reported to be contaminated with aflatoxins at levels above the recommended limits for total aflatoxin by the World Health Organization (Odda *et al.*, 2018). Although the aflatoxin contamination has been reported elsewhere in Uganda (Omara *et al.*, 2020), there is no scientific data concerning mycological and aflatoxin contamination of herbal medicine in northern Uganda. By investigating the prevalence and aflatoxin contamination levels in herbal medicine sold in Lira and Gulu cities, this study aims to inform evidence-based interventions to protect public health and promote the safe use of herbal medicines.

1.3 General objective

The study aims to assess the fungal and aflatoxin contamination levels of herbal medicine consumed in northern Uganda.

1.4 Specific objectives

- i To determine the level of aflatoxin in herbal medicines sold in Lira and Gulu cities.
- ii To ascertain the health risks associated with the consumption of herbal products.
- iii To identify the fungal species in the herbal medicine sold in Lira and Gulu cities.

1.5 Research questions

- i What are the levels of aflatoxin contamination in herbal medicines sold in Lira and Gulu cities?
- ii What are the potential health risks associated with the consumption of aflatoxin-contaminated herbal medicines in Gulu and Lira cities?
- iii What are the predominant fungal species contaminating herbal medicines sold in Lira and Gulu cities?
- iv What is the relationship between aflatoxin contamination level and herbal medicine moisture content?

1.6 Scope of the study

The study aims to assess the prevalence and levels of fungal and aflatoxin contamination in herbal medicines consumed in Lira and Gulu cities, northern Uganda. Specifically, the study aims to quantify aflatoxin levels in herbal medicinal products, evaluate the associated health risk, and identify fungal species present in the herbal medicine. To achieve these objectives, we collected samples from various herbal dealers in both cities, analyze them using laboratory techniques such as ELISA, and statistically analyze the results to determine the extent of contamination and associated health risks. By conducting these research, the study sets to provide valuable insights into safety and quality of herbal medicines in the region, contributing to efforts to improve public health. The study was conducted from March to June 2024.

1.7 Significance of the study

Since most people believe natural products are extremely safe for human consumption, the study will help raise public awareness regarding the potential risks associated with consuming herbal products. The study's findings can be used as a reference for establishing the permissible level of aflatoxin in herbal medicinal products consumed in northern Uganda.

1.8 Justification

Uganda is committed to advancing the third sustainable development goal (SDG) of "good health and well-being". Nonetheless, consuming herbal medicine contaminated with aflatoxin poses a significant health threat due to its carcinogenic, mutagenic, and teratogenic properties. Without appropriate intervention measures to tackle the health concern, attaining the goal is unfeasible. Therefore, this study, which aims to determine the aflatoxin levels in herbal medicine, is crucial in raising public awareness and providing evidence for necessary intervention measures.

There is still a lack of knowledge regarding fungal and aflatoxin contamination of herbal medicine in northern Uganda. This could be due to limited scientific research about aflatoxin and inadequate monitoring systems. This study will therefore provide scientific evidence to help regulate and monitor the consumption of herbal products contaminated with aflatoxin to achieve good health and well-being.

A considerable number of the rural Ugandans examined were exposed to aflatoxin daily, with many exhibiting elevated levels (Asiki *et al.*, 2014). Moreover, high aflatoxin exposure impacts more than 90% of the population in Uganda, as indicated by the detection of aflatoxin in their body fluids (Kang *et al.*, 2015). Consequently, it's crucial to better understand the sources of exposure in order to implement effective and long-lasting interventions. This study will thus provide evidence of additional potential sources of aflatoxin exposure beyond the staple cereals, as documented by other researchers.

CHAPTER TWO: LITERATURE REVIEW

2.1 Aflatoxin classes

Aflatoxins are highly oxygenated, polysubstituted coumarins with indiscernible structural differences. Chemical characterization of at least 18 different types of aflatoxins has identified six significant ones as: aflatoxin B₁ (AFB₁), aflatoxin B₂ (AFB₂), aflatoxin G₁ (AFG₁), aflatoxin G₂ (AFG₂), aflatoxin M₁ (AFM₁), and aflatoxin M₂ (AFM₂) (Benkerroum, 2020a). The structure of the six significant aflatoxin are shown in figure 4.1. The G-series (six-membered lactones) fluoresce yellow-green on thin-layer chromatography plates, but the B- aflatoxins, mainly pentanone derivatives, exhibit strong blue fluorescence under UV light, thus the B and G designations. Dihydroxy derivatives of AFB₁ and AFG₁ are AFB₂ and AFG₂. When AFB₁ is absent, the other aflatoxins are usually not detected (Chun *et al.*, 2007). The M series is named after harmful metabolic by-products of the B series that fluoresce blue-violet and have been found in the milk of animals fed feed contaminated with aflatoxin. In aflatoxin nomenclature, major and minor are denoted by the subscripts 1 and 2, respectively.

Table 2. 1: A summary of the main aflatoxins that the Aspergillus species of mould produce; (adopted from Bbosa et al. (2013))

Difuranocoumarins	Type of aflatoxin	Aspergillus species
	Aflatoxin B ₁ (AFB ₁)	<i>A.flavus, arachidicola, bombycis, minisclerotigenes, nomius, ochraceoroseus, A. parasiticus, A. pseudotamarii, A.rambellii, Emericella venezuelensis</i>
	Aflatoxin B ₂ (AFB ₂)	<i>A. arachidicola, A.flavus, A. minisclerotigenes, A. nomius, A. parasiticus</i>
	Aflatoxin B _{2A} (AFB _{2A})	<i>A. flavus</i>
	Aflatoxin M ₁ (AFM ₁)	<i>A. flavus, A. parasiticus</i> ; metabolite of aflatoxin B ₁ in humans and animals and comes from a mother's milk.
	Aflatoxin M ₂ (AFM ₂)	Metabolite of aflatoxin B ₂ in milk of cattle fed on contaminated foods.
	Aflatoxin M _{2A} (AFM _{2A})	Metabolite of AFM ₂
	Aflatoxicol (AFL)	<i>A. flavus</i> , metabolite of aflatoxin B ₁
	Aflatoxicol M ₁	Metabolite of AFB ₁
Difurocoumarolactone	Aflatoxin G ₁	<i>A. arachidicola, A.flavus, A.minisclerotigenes, A.</i>

series	(AFG ₁)	<i>nomius</i> , <i>A. parasiticus</i>
	Aflatoxin G ₂ (AFG ₂)	<i>A. arachidicola</i> , <i>A. flavus</i> , <i>A. minisclerotigenes</i> , <i>A. nomius</i> , <i>A. parasiticus</i>
	Aflatoxin G _{2A} (AFG _{2A})	Metabolite of AFG ₂
	Aflatoxin GM ₁ (AFGM ₁)	<i>A. flavus</i>
	Aflatoxin GM ₂ (AFGM ₂)	Metabolite of AFG ₂
	Aflatoxin GM _{2A} (AFGM _{2A})	Metabolite of AFGM ₂

The fungus *A. flavus* and *A. parasiticus* fungi are the main producers of over 20 similar fungal metabolites known as aflatoxins. Aflatoxins belong to a class of difuranocoumarins known as the difurocoumarocyclopentenone series (AFB₁, AFB₂, AFB_{2A}, AFM₁, AFM₂, AFM_{2A}, and aflatoxicol) and the difurocoumarolactone series (AFG₁, AFG₂, AFG_{2A}, AFGM₁, AFGM₂, AFGM_{2A}, and AFB_{2A}) (Ibrahim & Menkovska, 2018).

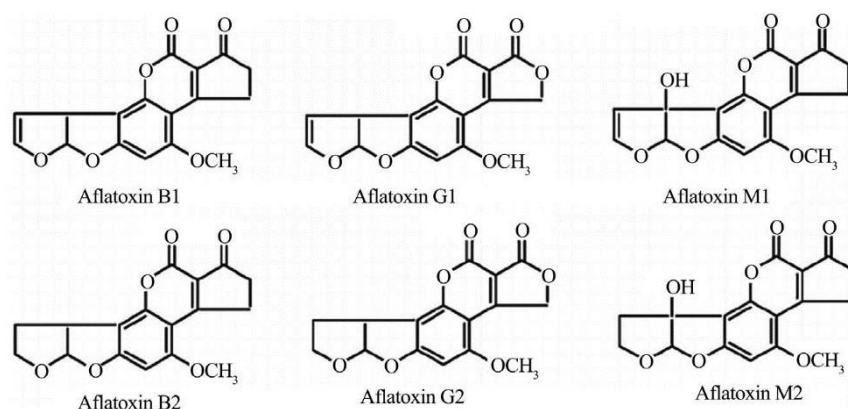


Figure 2. 1: Chemical structure of aflatoxins B1, G1, M1, B2, G2, M2; (adopted from Ibrahim & Menkovska, 2018)

2.2 Aflatoxin in herbal medicine

The quality and safety of herbal products are closely related to compliance with the principles of good agricultural practice at every stage of production (WHO, 2003). To safeguard the consumers who use these items to better their health, plants that are designated as medical materials should be carefully preserved and tested for aflatoxins (Ałtyn & Twarużek, 2020). The dose of herbal intake depends on the consumer's health.

Thus, the level of aflatoxin contamination varies; therefore, the level of aflatoxins should be considered individually for each type of medicinal plant (Pallarés *et al.*, 2022). One of the challenges of international trade is the lack of common guidelines and a monitoring body to enforce the regulation of aflatoxin in herbal plants and their products, especially for dietary supplements (Pallarés *et al.*, 2022). Aflatoxins are a consequence of fungal growth and, therefore, also an indicator of hygienic deficits during production and storage of herbal medicine (Ałtyn & Twarużek, 2020). Generally, drying within 24 hours of harvest to reduce the moisture content to 14% or below inhibits the growth and aflatoxin production by *Aspergillus*, the producer of aflatoxin (de Sousa Lima *et al.*, 2020).

Aflatoxin contamination of herbal medicine is a widespread problem worldwide. According to Qin, *et al.* (2020) a significant proportion (23.4%) of traditional Chinese herbs tested between 2000 and 2018 were contaminated with aflatoxins, with levels detected in 697 out of 2979 batches ranging from 0.02 to 1268.8 µg/kg. Another study by Zhao *et al.* (2016) reported that 14 of 22 samples were contaminated with at least one type of aflatoxin at concentrations ranging from 0.2 to 7.5 µg/kg. A comparable study carried out in South Korea found that 58 of 700 samples of herbal remedies tested positive for aflatoxin, with 6 and 10 of those samples exceeding the regulatory limits for total aflatoxin B₁ and aflatoxin B₁ in South Korea, which are 15 and 10 g/kg, respectively (Shim *et al.*, 2012).

A study conducted by (Keter *et al.*, 2017) investigated the risk of fungi, specifically aflatoxin and fumonisin, in medicinal herbal products in Kenya. The alarming results showed that 69% of the sampled products exceeded the acceptable fungal limits set by international Pharmacopeia. Aflatoxin levels varied from 1 to 24 parts per billion (ppb), while fumonisin levels ranged from 1 to over 20 ppb. In another study conducted in Nigeria by Ezekwesili-Ofili *et al.* (2014) assessed the bio-load and aflatoxin content in herbal medicines from various Nigerian states. Notably, Aflatoxin B₁ was the predominant aflatoxin, with 18.6% of samples surpassing the permissible limits established by the World Health Organization.

2.3 Fungi producing aflatoxin

A. flavus, *A. parasiticus*, *A. nomius*, and *A. tamarii*, all soil borne fungi responsible for the degradation of plant materials, are the principal producers of aflatoxins (Bennett *et al.*, 2003). Aflatoxin production by about 20 *Aspergillus* species has been documented, although research on additional, unique, and potentially aflatoxin-producing fungi is ongoing (Norlia *et al.*, 2019). Most species produce B-type aflatoxins via the polyketide pathway as

difuranocoumarin derivatives. However, species related to *A. parasiticus*, *A. nomius*, *A. toxicarius*, *A. bombycis*, *A. parvisclerotigenus*, *A. minisclerotigenes*, and *A. arachidicola* can additionally produce G-type aflatoxins (Varga *et al.*, 2009). AFB₁, AFB₂, AFB_{2A}, and AFG_{2A} have been isolated from cultures of *A. flavus* and *A. parasiticus*, while AFGM, parasiticol, and aflatoxicol are solely produced by *A. flavus* (Syamilah *et al.*, 2022).

Wei *et al.* (2023) reported that herbal medicines were found to be extensively contaminated with aflatoxins (62.92%), and it was primarily attributed to the dominant fungal genus *Aspergillus* (43.77%). Further analysis revealed 20 fungal species, mostly *Aspergillus* and *Fusarium*, which are capable of producing harmful aflatoxins and ochratoxin. In another study conducted in China, a total of 173 fungal isolates were identified, consisting of 24 genera, with *Fusarium* (27.74%) and *Alternaria* (20.81%) being the most prevalent (Wang *et al.*, 2024). This is attributed to the cultivation of herbs in soil which predisposes them to fungal infestation, while post-harvest handling, preparation, storage, and transportation create opportunities for additional contamination by various pathogenic fungal species, leading to mould development and aflatoxin synthesis. A different study done in Brazil by Bugno *et al.* (2006), evaluated fungal contamination in 91 medicinal plant samples and revealed that *Aspergillus* and *Penicillium* genera accounted for 89.9% of isolates, with 21.97% demonstrating mycotoxigenic potential. Specifically, 42.9% produced aflatoxins, 22.4% produced ochratoxin A, and 34.7% produced citrinin. These findings highlight the urgent need for standardized regulations governing toxigenic mould limits in herbal products to safeguard consumer health.

2.4 Health effects of aflatoxin

Aflatoxicosis is a disease caused by aflatoxins in both humans and animals (Dhanasekaran *et al.*, 2011). Acute primary aflatoxicosis, resulting from the consumption of moderate to high levels of aflatoxins, manifests in two main forms (cirrhosis or carcinoma of the liver). Hemorrhage, severe liver damage, oedema, changes in digestion, nutrient absorption, and/or metabolism, and even death are only caused by specific acute disease episodes. AFB₁ has been linked to epidemics of acute hepatic damage (Dhanasekaran *et al.*, 2011). Globally, there is evidence of acute aflatoxicosis in humans, particularly in nations like Taiwan, Uganda, India, Kenya, and many others (Williams *et al.*, 2004). Ingestion of low to moderate levels of aflatoxins results in chronic primary aflatoxicosis (Sarma *et al.*, 2017). The mutagenic effects of aflatoxins, which result in changes (mutations) to the genetic code via altering DNA, are among the chronic types of aflatoxicosis. Teratogenic effects are

associated with congenital malformations (Benkerroum, 2020). Chromosome breakage, chromosome piece rearrangements, chromosome gain or loss, and gene alterations are a few of these modifications.

The genotoxic effect, where electrophilic carcinogens alter genes through interactions with DNA and hence become a potential cause of DNA damage, and the genotoxic carcinogens (Benkerroum, 2020). Aflatoxin's long-term effects have been shown to impair normal immunological responses by either lowering phagocytic activity or T cell number or function, as was seen in an animal model (Williams *et al.*, 2004). A dose-response study of aflatoxin exposure and growth rate in babies and children revealed that aflatoxins can interfere with nutrition (Gong *et al.*, 2016). In animal models, aflatoxins also modify nutrients like vitamin A or D, rendering them unavailable to the body's normal physiology and resulting in nutritional deficits (Seid & Mama, 2019).

As indicated in figure 2.2, aflatoxin contamination in foods and feeds can significantly impact human and animal health (Dhanasekaran *et al.*, 2011). More than five billion people in developing nations are at risk of chronic aflatoxin exposure due to the consumption of contaminated foods (Williams *et al.*, 2004). Over four billion people are thought to acquire liver cancer linked to aflatoxin, particularly hepatocellular carcinoma. Exposure to aflatoxins is primarily an issue in developing and underdeveloped nations because of insufficient food storage and processing regulations, coupled with high malnutrition rates (Williams *et al.*, 2004).

Aflatoxins have also been linked with kwashiorkor and marasmus in children in most sub-Saharan countries (Mupunga *et al.*, 2017). Studies on humans have shown that aflatoxins raise circulating alpha tumour necrosis factor levels, indicating that these aflatoxins may be potentially immunotoxic to humans. Aflatoxin exposure also risks fetuses, as breast milk passes from mother to child (Gong *et al.*, 2016). In addition, aflatoxins have been linked to Reye-like Syndrome occurrences in countries such as Thailand, New Zealand, Czechoslovakia, the United States, Malaysia, Venezuela, and Europe, (World Health Organization, 2000). Symptoms of acute toxicosis in dairy and beef cattle include anorexia, depression, a significant reduction in milk production, weight loss, lethargy, and gastrointestinal dysfunctions such as ascites, icterus, tenesmus, abdominal pain, and bloody diarrhea, along with decreased feed intake and efficiency.

Additional symptoms include weight loss, jaundice, abortion, hepatoencephalopathy, blindness, walking in circles, ear twitching, and frothy mouth (Agag, 2004). Poultry may exhibit inappetence, weight loss, reduced egg production, leg and bone complications, poor pigmentation, fatty liver, kidney dysfunction, bruises, and death, a reduction in natural immunity and increased susceptibility to parasitic, bacterial, and viral infections (Agag, 2004).

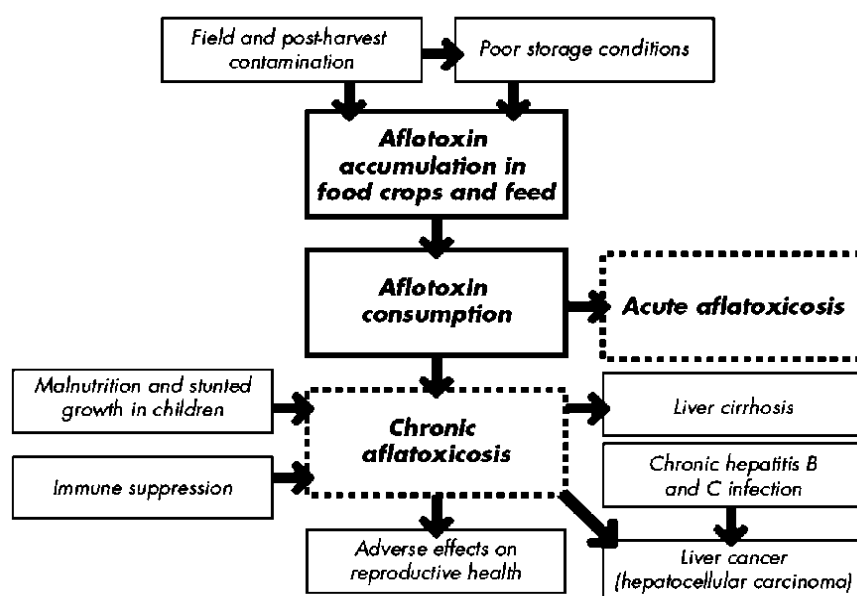


Figure 2. 2: Aflatoxin disease pathways in humans: adopted from (Wu & Khlangwiset, 2010).

2.5 Economic effect of aflatoxin

Aflatoxin contamination in Sub-Saharan Africa has led to the rejection of commodities in the international market, with projected annual financial losses of up to \$25.8 million (Wu & Munkvold, 2008). A report from the Partnership for Aflatoxin Control in Africa indicates that aflatoxin contamination lowered economic growth in Uganda by 0.26% due to decreased productivity brought about by illnesses related to aflatoxin exposure (PACA, 2017). According to estimates, the value of agricultural exports declined by 43.5% of the overall exports (\$37.56 million), while grain exports fell by 45% of the entire total (Lukwago *et al.*, 2019). Household consumption and household disposable income declined by US\$59.1 million and US\$79.3 million, respectively. Export rejections due to aflatoxin result in an estimated annual loss of 38 million US dollars for Uganda (Ankwasa *et al.*, 2021). Furthermore, 3700 cases of liver cancer attributed to aflatoxin cost Uganda \$577 million annually. Aflatoxin-related illnesses also cost the Ugandan government an additional US \$910,00,000 in healthcare expenditures (Ankwasa *et al.*, 2021).

In addition, more than 600,000 metric tonnes of maize valued at \$10 billion Uganda shillings was rejected by Kenya due to aflatoxins above the permissible levels (Lukwago *et al.*, 2019).

2.6 Aflatoxin in Uganda

Aflatoxin contamination in the key food crops such as maize, groundnuts, and sorghum is high and widespread in Uganda (Omara *et al.*, 2020). The districts with the highest risk for aflatoxin contamination in sorghum were Soroti, Amuria, and Lira, where up to 100% of the sorghum had aflatoxin levels exceeding the Ugandan regulatory standard of 10 ppb (PACA report, 2017). In Uganda, Soroti district is the leading producer of sorghum and groundnuts, with research indicating that up to 20% of groundnuts from Soroti and Iganga exhibit unacceptable levels of aflatoxins, making these districts the most at risk (PACA report, 2017). In a study conducted in Northern Uganda by Echodu *et al.* (2019) revealed that all 105 food samples analysed contained detectable levels of total aflatoxin. A similar study by Wokorach *et al.* (2021) found that more than 50% of the grains obtained from the districts of Kitgum, Kole, Lamwo, Oyam, and Pader were contaminated with aflatoxins.

2.7 Risk factors associated with aflatoxin

Numerous studies have shown the extreme toxicity of aflatoxins, which include immunosuppression, hepatotoxicity, nephrotoxicity, and reproductive problems. Aflatoxins are also mutagenic, teratogenic, and carcinogenic; exposure to such substances poses a major risk to human health (Dhakal & Sbar, 2021). AFB₁ may induce teratogenic effects because research indicate it can suppress the immune system, disrupt foetal development, and alter cell differentiation. AFB₁ exposure potentially impacts the liver tissues of both humans and animals. Severe exposure to AFB₁ can result in liver cancer and possibly death (Benkerroum, 2020).

Risk assessment is crucial for ensuring the safety of herbal medicines and products containing fungal spores (PFS). Studies have reported calculated dietary exposure using estimated daily intake (EDI) and assessed risk characterization using cancer risk and margin of exposure (MOE) approaches (Ab Dullah *et al.*, 2023). Research revealed varying AFB₁ exposure levels worldwide, with China having the highest exposure (88.27 ng/kg bw/day), followed by Taiwan (41.19 ng/kg bw/day), South Korea (7.34 ng/kg bw/day), Egypt (5.22 ng/kg bw/day), Turkey (3.59 ng/kg bw/day), Morocco (2.908 ng/kg bw/day), Thailand (0.79 ng/kg bw/day) and Indonesia (0.07 ng/kg bw/day) (Jeyaraj *et al.*, 2022). The Measure of exposure (MOE) approach is suitable for characterizing carcinogenic and genotoxic

substances. A MOE value of 10,000 or higher is considered a low priority for risk management action (Ab Dullah *et al.*, 2023).

2.8 Mitigation strategies

International, regional, and statutory efforts: in Uganda, efforts have been made to minimise aflatoxin by raising national awareness. The Eastern Africa Grain Council (EAGC) and Uganda National Bureau of Standards (UNBS) are working together on this through the Eastern Africa Grain Institute. In order to understand the integrated East African maize standard (EAS 2: 2013), food standardization, comparison of East African standards with international standards, standard maize sampling techniques, maize grading, aflatoxins, and the available methods for aflatoxin analysis, maize exporters, traders, farmer-based organizations, and warehouse handlers received training between 2015 and 2018 (New Vision, 2018). In collaboration with Makerere University, the National Agricultural Research Organization (NARO) developed a manual for managing aflatoxin in peanuts in 2010. The manual provides a broad review of aflatoxins (structures, health effects, and economic implications), discusses control measures for aflatoxins, and lists specific farming methods used in Uganda that encourage the formation of aflatoxins.

It was developed specifically to give detailed guidelines on reducing aflatoxin contamination in peanuts and increasing the value of groundnuts and their products (New Vision, 2015).

Planting and breeding crop varieties less prone to fungus development is recommended. One of the most effective methods for lessening the impact of aflatoxin-producing fungus species has been reported. Studies have revealed that some local maize cultivars have lower aflatoxin levels than imported varieties, indicating that native cultivars of crops resistant to aflatoxins demand further investigation. Serenut 2 is a peanut variety reported to be genetically more resilient to aflatoxins and fungus growth in Uganda (Kalule, 2010). Crop cultivars that are tolerant of or resistant to disease, pests, and drought have been proven to significantly lower aflatoxin contamination (Kebede *et al.*, 2012). More specifically, host and parasite macro- and micro molecular trafficking have raised the prospect of using cross-species RNA interference to get around the aflatoxin issue. This prevents aflatoxin accumulation by supplying maize with molecules that stop aflatoxin biosynthesis during infection with an aflatoxigenic fungus (Mutungi *et al.*, 2019).

A workable approach to reducing aflatoxin is to promptly harvest grains in dry conditions when they are fully mature and to remove any damaged maize kernels or cobs as soon as

possible (Mutungi *et al.*, 2019). It has been reported by Omara *et al.* (2020) that visual sorting, winnowing, washing, crushing, and dehulling can reduce the levels of aflatoxin in grains by up to 40–80%. Sorting is strongly advised for lowering the amount of aflatoxin in food, particularly in peanuts and beans. However, after sorting the mouldy peanuts, they should not be given to children (known as "lake" in northern Uganda) or used to make peanut paste. Clean water can be used for sorting; damaged seeds or grains float while good ones sink and can be cooked. Malting, roasting, and soaking and heating in 'magadi' soda are some additional techniques that have been utilised to lower the amounts of aflatoxins in maize (Omara *et al.*, 2020). Unfortunately, the rural Lango sub-region community unknowingly uses *magadi* as a catalyst to speed up the cooking of vegetables, beans, peas, and sesame-based foods ('*alakena*' and '*agwaca*'). Aflatoxin control relies heavily on crop protection from insect attacks. This can be accomplished by utilising ash while in storage, just like it is done with maize, and planting essential oils, like *Eucalyptus saligna*, which has been shown to have bio-insecticidal activity (Omara *et al.*, 2020).

Biocontrol strategies employing concoctions from plants have been investigated and reported to inhibit *A. flavus* mycelial growth and proliferation (Gasperini *et al.*, 2019). Essential oils of *Azadirachta indica* (neem) and *Morinda lucida* have been exported to retard aflatoxigenic *A. flavus* growth and its aflatoxin biosynthesis potential in inoculated maize grains. *Aframomum danielli* (*Zingiberaceae*) powder regulates mould and insect infestation in maize and soybeans in storage for over a year under ambient conditions (Gasperini *et al.*, 2019). Competitive exclusion has been reported as a feasible aflatoxin control strategy (Ehrlich, 2014).

Harvested crops should be shelled and cleaned before storage to reduce incidences of pest infestation, which may promote aflatoxigenic contamination (Kaaya & Kyamuhangire, 2006). Aflatoxin-growth-friendly temperatures between 25°C and 32°C and persistent relative humidity above 65% should not be reached in storage facilities; thus, they should also be well ventilated (Kaaya & Kyamuhangire, 2006). According to Grain and Toxins Ltd. of Uganda, the use of the "Toxin Scrub," a postharvest aflatoxin removal innovation, has been restricted by its exorbitant cost. To remove nearly all of the aflatoxins from the grain, the method makes use of ozone, a potent oxidizer. Aflatoxin instability to UV radiation in the presence of oxygen, pH extremes (3, >10), and oxidising chemicals including sodium hypochlorite, potassium permanganate, chlorine, hydrogen peroxide, ozone, and sodium perborate offer evidence for this (Kaaya & Kyamuhangire, 2006).

No scientifically proven specific antidote for ingested aflatoxins has been reported. However, the timely use of l-methionine (200 mg/kg) and sodium thiosulfate (50 mg/kg) every 8 hours has reported therapeutic significance. In addition, dietary intake of protein, vitamins, and antioxidants can be encouraged in cases of aflatoxicosis (Ráduly *et al.*, 2020).

2.9 Summary/gaps in literature

Medicinal herbs are highly susceptible to toxigenic fungal infections and aflatoxin contamination, which may arise during either the pre- or post-harvest stage due to poor growing conditions, inadequate drying, or storage in warm, humid conditions (Qin *et al.*, 2020; Zhang *et al.*, 2018). The possibility of medicinal herbs and related agricultural products exhibiting toxic effects due to aflatoxin contamination is garnering increasing attention worldwide (Zhang *et al.*, 2018). In Africa, as much as 80% of the population relies on traditional medicine for primary health care (WHO, 2022). Lira and Gulu cities were chosen because of the many herbal dealers and high population compared to the other districts and municipalities within the region and there is widespread consumption of herbal medicine. Despite the widespread use of herbal medicine, limited studies have assessed the fungal and aflatoxin contamination of herbal medicines sold in Northern Uganda.

CHAPTER THREE: MATERIALS AND METHODS

3.1 Study area

The study was conducted in Gulu ($2^{\circ}46'54.0''\text{N}$ $32^{\circ}17'57.0''\text{E}$) and Lira ($2^{\circ}15'29.0988''\text{N}$ $32^{\circ}53'14.6652''\text{E}$) cities, northern Uganda (Figure 3.1).

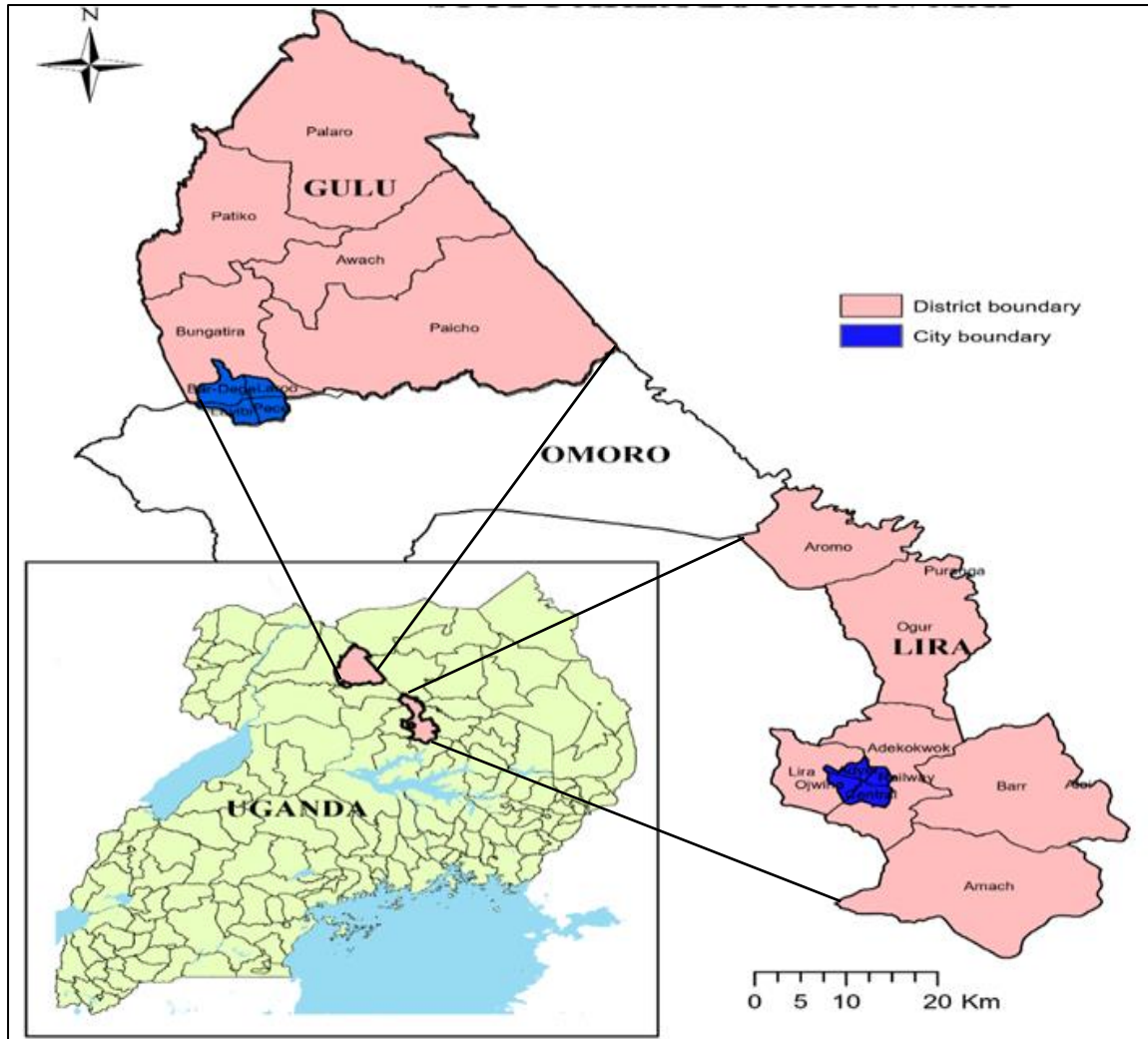


Figure 3. 1: Location of the studied areas in northern Uganda

Gulu City is bordered to the south by Omoro district, east by Pader district, and west by Nwoya district. Similarly, Lira city is bordered by Dokolo to the south, Pader to the north, Kole to the west, and Alebtong to the east. According to UBOS (2024), the population of Lira and Gulu cities is 246,437 and 232,723, respectively. Lira City's main economic activities are agro-processing and small and medium enterprises, according to the Lira City Development Plan (2022).

Gulu City's economy is based on the wholesale and retail trades, the construction industry, and non-agricultural household-based enterprises, as stated in the Gulu City Development Plan (2022). In both cities, major sources of employment include commerce, trade, industry, and public services. Lira and Gulu cities have an annual average temperature of 25.02 °C and 25.48 °C, respectively. The area has two rainfall periods, wet and dry, with the wet season starting in March and lasting through November annually. The rainfall pattern is unimodal, peaking in May, June, July, and August, with an average annual rainfall between 600 and 1200 mm. The dry season starts in November and lasts until March (Uganda National Meteorological Authority, 2022).

3.2 Study design

The research was a cross-sectional study that was conducted between March and June 2024.

3.3 Population

The target study population comprised all herbal dealers within the two cities.

3.4 Sample size

Samples were obtained from six herbal dealers from Lira City and seven herbal dealers from Gulu City. Only four samples were obtained from each herbal dealer, totalling 52 samples of herbal medicines. The small number of samples collected was attributable to constrained financial resources.

3.5 Sampling technique and sample collection

Uganda's traditional herbalist association in the two cities was contacted to assist in identifying herbal dealers. The snowball sampling technique was used to recruit herbal dealers. From each herbal dealer in each city, at least four commonly available herbal medicines in powder form were purchased. Lira and Gulu cities were chosen because of the many herbal dealers and high population compared to the other districts and municipalities within the region. Samples drawn from each herbal medicine were put in separate sterilized polythene bags with specific sample identifiers and transported to the Gulu University Multifunctional Research Laboratory for further analysis.

3.6 Inclusion criteria

To participate, herbal dealers needed to be at least 18 years old with at least a minimum of one year's experience in selling over five different herbal products, and must have been resident of either Lira or Gulu City. Only commonly used herbal medicines sourced from herbal dealers within the cities were used, provided they were of sufficient quantity for laboratory analysis.

3.7 Exclusion criteria

Herbal dealers who are under 18 years of age were not eligible to participate. Moreover, those with less than one year of experience in selling herbal products were excluded. Additionally, herbal dealers residing outside of Lira and Gulu cities were excluded. Participants who did not meet the requirement of selling a minimum of five different herbal products were also excluded. Adulterated herbal samples containing synthetic and non-botanical ingredients or samples that had undergone extensive processing, such as those in capsule and tablet form, were excluded from the study.

3.8 Determination of the moisture content

The moisture content of samples was determined using the method described by Cauvain and Young (2008). Firstly, the crucible was weighed (M_1), and then approximately 3 g of the sample was added to it and weighed again (M_2). Next, the crucible and sample were placed in an oven set at 105°C for 3 hours, allowed to cool in a desiccator, and weighed again (M_3). Finally, the moisture content of the sample was calculated using the equation 1 below.

Moisture content, w .

$$\frac{M_2 - M_3}{M_2 - M_1} \times 100 \dots\dots\dots \text{Equation 1}$$

Where: M_1 is the mass of the crucible,

M_2 is the mass of the crucible plus the sample,

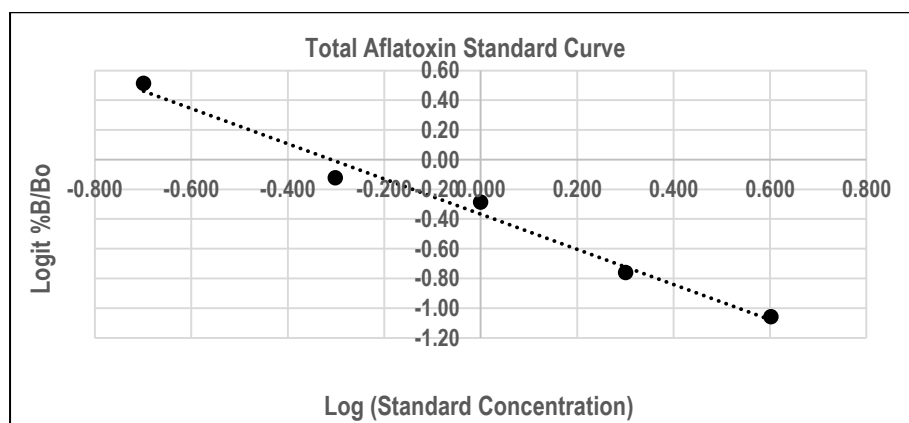
M_3 is the mass of the crucible plus the dry sample.

3.9 Extraction of aflatoxin

Aflatoxins in the herbal medicine were extracted using a 70:30 v/v methanol: water solution following the methods of Wacoo *et al.* (2014). Briefly, 1.5 g of the powder was mixed with 1.5 mL of methanol: water (70:30 v/v) and shaken at 300 rpm for 10 min using a rotary shaker. The mixture was filtered using Whatman No. 1 filter paper after being allowed to settle for 10 minutes. Using the filtrate, total aflatoxin was determined by Helica Total Aflatoxin Rapid ELISA Test Kit.

3.10 Determination of aflatoxin

The resultant filtrate was processed with the Helica Total Aflatoxin Rapid ELISA Test Kit reagents following the manufacturer's instructions. A microwell reader fitted with a 450 nm filter was used to measure the optical density of the reaction mixture in each microwell. A standard curve (figure 3.2) was constructed using ELISA absorbance readings of the total aflatoxin standards (0.0, 0.2, 0.5, 1.0, 2.0, 4.0 ng/mL) to determine aflatoxin concentrations in parts per billion (ppb). The concentrations of total aflatoxin in the samples were determined by interpolating from a standard curve (figure 3.2). This curve was constructed by plotting the average optical density (OD) values, expressed as a percentage of the zero-standard OD (%B/B₀), against the corresponding aflatoxin concentrations of the standards.



$$R^2: 0.983897$$

$$\text{Slope: } -1.18507$$

$$\text{Intercept: } -0.36777$$

Figure 3. 2: Aflatoxin standard curve

3.11 Health risk assessments

3.11.1 Estimation of aflatoxin daily intake

The EDI was calculated using the total aflatoxin concentration obtained in the herbal medicines, the daily herbal intake as prescribed by the herbal dealer, and the mean body weights of 11.3 kg and 72.3 kg/person for children and adults, respectively (Ralston & Myatt, 2016). The EDI expressed in µg/kg of the body weight per day (µg/kg bw per day) was calculated using **Equation 2** (Ishikawa *et al.*, 2016).

$$EDI = \frac{\text{Daily intake of herbal medicine} \times \text{total aflatoxin concentration}}{\text{average body weight in kilograms}} \dots\dots\dots \text{Equation 2}$$

3.11.2 Hazard quotient

The Hazard Index (HI) was determined by dividing the EDI by TD50 (the daily dose (µg/kg/body weight/day) at which 50% of test animals would have developed tumours), divided by a safety factor of 50,000 as described by Ishikawa *et al.* (2016). TD₅₀ is the dose (µg/kg/bw/day) required to induce tumours in half of the test animals that would have remained tumour-free at zero doses. The complete mathematical relationship between these parameters is given in **Equation 3**.

$$\sum_{n=0}^i \frac{EDI/TD50}{50,000} \dots\dots\dots \text{Equation 3}$$

3.12 Characterisation of the fungus

3.12.1 Identification of Fungal isolates

The powdered samples were mixed with 1mL of distilled water and later inoculated in duplicates on potato dextrose agar (PDA, 40 g/L) media and incubated at 30 °C for 5 days. The fungal growth was sub-cultured on a new PDA medium. The sub-cultured isolates were grown at 30 °C for 10 days to generate spores. The fungal growth was identified macroscopically based on the colony morphology (Okayo *et al.*, 2020).

3.12.2 DNA extraction

The fungal DNA was extracted as described by Wokorach *et al.* (2022). The potato broth containing spores in the Eppendorf tube was centrifuged at maximum speed for 5 minutes. The potato broth was discarded, leaving the fungal spores in the tube. The fungal spores were picked and placed in a clean mortar. 500 µl of CTAB buffer was added to the sample in the

mortar. The sample was ground using the pestle until it was a fine and milky, or cloudy, solution. The sample mixture was placed back in the Eppendorf tube and 10 µl of proteinase K was added. The sample mixture was vortexed thoroughly and then incubated in a warm water bath at 65 °C for 30 minutes. 500 µl of chloroform isoamyl alcohol (24:1) was added to the reaction mixture, vortexed till milky, and then centrifuged at 1300 rpm for 15 minutes. The upper aqueous phase (300 µl) was transferred to a fresh collection tube, and 300 µl of cold (-20°C) isopropanol was added to the reaction mixture.

The mixture was vortexed and centrifuged for 15 minutes to allow for the formation of DNA pellets. The supernatant was discarded to leave the precipitated DNA at the bottom of the tube. The precipitate was washed with 150 µl of 70% ethanol and centrifuged at 13000 rpm for 2 minutes. The alcohol was removed, leaving the precipitate in the tube. The tube was left open for 10 minutes to air-dry the ethanol. The pellet was re-suspended in 50 µl of molecular water nuclease-free water. The DNA was stored at -20 °C, and PCR amplification was done to confirm the fungal species as described by Wokorach *et al.* (2022).

3.12.3 DNA amplification

DNA amplification was done using primers ITS1F (5'-CTTGGTCATTTAGAGGAAGTAA-3') and ITS4R (5'-TCCTCCGCTTATTGATATGC-3') that target the Internal Transcribed Spacer gene were used (Jurjević *et al.*, 2015). The PCR mix consisted of the following reagents: 5µL of GoTag PCR buffer (5X), 1.25µL of dNTP (10 mM) solution mix, 1µL (5 nM) forward primers, 1µL (5 nM) reverse primers, 0.125µL of GoTaq polymerase (5 U/µL), 2µL of DNA templates, and 14.625µL of nuclease-free water. The SimpliAmp™ Thermal Cycler (Applied Biosystems™ by Life Technologies) was used to carry out the PCR amplifications under the following amplification conditions: initial denaturation at 95 °C for 3 min (1 cycle), 35 cycles of 1-min actual denaturation at 94 °C, 30 s of primer annealing at 50 °C, and 45 s of extension at 72 °C. Final extension at 72 °C for 5 min. Nuclease-free water was used as a no template control during the PCR amplification. The quality of the amplicons was determined by gel electrophoresis using 1% agarose gel. The PCR amplicons were sent to South Africa (Inqaba Biotech laboratory) for purification, and later unidirectional sequencing was performed.

3.13 Data analyses

The statistical analysis for this study was performed using SPSS version 25. The data on aflatoxin was categorized based on the city of origin, and the prevalence was determined by

dividing the total number of herbal medicines positive for aflatoxin by the total number of samples tested, then multiplying by 100. The Shapiro-Wilk test was used to check the data on aflatoxin concentration for normality, whereas the Kruskal-Wallis test was used to compare the level of aflatoxin contamination in the herbal products. A Dunn-Bonferroni test was performed to mitigate the risk of Type I error due to multiple testing. A Mann-Whitney U test assessed possible differences in aflatoxin contamination levels between the two cities. The mean moisture content percentages of herbal samples from Gulu and Lira cities were statistically compared using an independent samples t-test. A Spearman rank correlation was performed to determine the association between moisture content and aflatoxin contamination in medicinal herbal products. The estimated daily intake and hazard quotient was calculated using Excel.

To identify the fungal species, the fungal isolates were assigned species names after comparison with representative sequences available in National Center for Biotechnology Information (NCBI). The obtained sequences were submitted to GenBank, and an accession number was assigned. For phylogenetic analysis, contigs obtained were analyzed using BioEdit 7.2.5 software and aligned using Clustal W of MEGA 11.0.13 software.

3.14 Ethical consideration

Ethical approval (GUREC-2023-621) was obtained from the Gulu University Research and Ethics Committee (GUREC). The privacy and confidentiality of the herbal shops were kept, and the data collected during the study was securely stored and handled in accordance with guidelines and regulations by the National Drug Authority (NDA). According to the Professional Guidelines for Regulation of Local Traditional/Herbal Medicines for Human and Veterinary Use in Uganda, Doc. No. DAR/GDL/033, Revision No. 0, ‘personnel entrusted with the handling of herbal materials, herbal preparations, and finished herbal products must maintain a high standard of personal hygiene’.

3.15 Assumptions of the study

The ITS gene region, known for its specificity in differentiating between various fungal species, was utilized in this study. Samples collected were representative of the herbal medicines sold in Lira and Gulu cities. Optimal laboratory conditions were maintained to facilitate accurate ELISA and DNA sequencing procedures. The samples were analysed and rigorously verified to minimize errors and ensure accuracy.

3.16 Limitations and delimitations of the study

Sensitivity of the ELISA kit may have limited the detection of low levels of aflatoxin in samples. Inefficient DNA extraction could have resulted in low-quality DNA, compromising subsequent PCR and sequencing. Suboptimal PCR amplification conditions might have affected the efficiency and specificity of the target ITS region. To address these limitations, the ELISA kit was validated using aflatoxin standards to ensure its sensitivity, specificity, and accuracy. Additionally, DNA extraction protocols were optimized to obtain high-quality DNA, and PCR conditions were optimized to enhance amplification efficiency and specificity.

CHAPTER FOUR: RESULTS

4.1 Aflatoxin contamination of herbal medicine

4.1.1 Moisture content of herbal medicinal product sold in Gulu and Lira city

Analysis of 52 herbal medicinal products revealed that 17 (32.7%) exceeded the recommended moisture content level of 13%. The box plot indicates that Gulu City has a higher average moisture content percentage compared to Lira City, with an outlier observed in Lira as shown in figure 4.1 below. Based on the t-test results, there is no significant difference ($p = 0.109$; t value = -1.636 ; $df = 46.205$) in the mean moisture content percentage between Gulu and Lira cities.

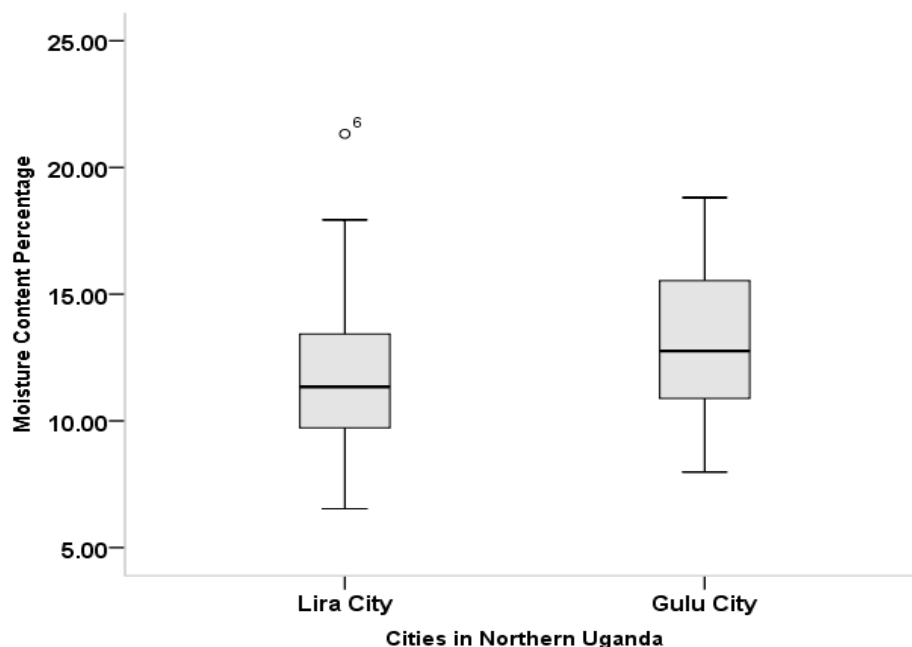


Figure 4. 1: A box plot showing the mean moisture content percentage in the two cities

4.1.2 Proportion of aflatoxin in herbal medicinal products

Of the 52 herbal medicine samples analysed, 18 were found to be contaminated with aflatoxin. The overall aflatoxin contamination percentage was 35%. Considering individual cities, Gulu City had a slightly higher prevalence of aflatoxin contamination (36%) compared to Lira City (33%) as shown in Table 4.1 below.

Table 4. 1: Proportion of Herbal medicine with aflatoxin in the Cities

City	N	Negative		Positive	
		Frequency	Percentage	Frequency	Percentage
Lira City	24	16	67	8	33
Gulu City	28	18	64	10	36
Total	52	34	65	18	35

Further analysis at the division level revealed varying contamination rates within each city. Lira City West had the highest contamination percentage (42%), while Lira City East had the lowest (25%). In Gulu City, Layibi-Bardege Division and Pece-Laroo Division had contamination percentage of 38% and 33%, respectively as shown in the figure 4.2.

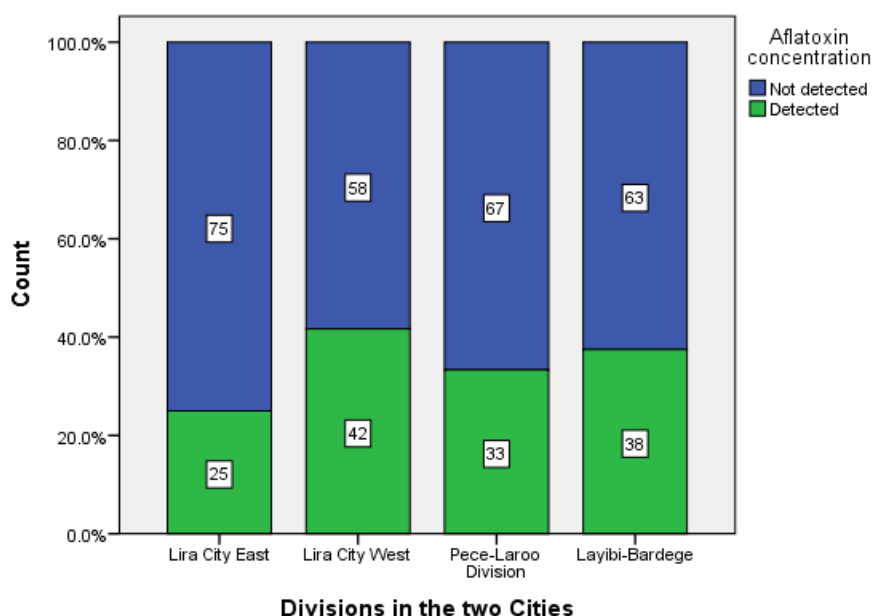


Figure 4. 2: A stacked bar graph showing the proportion of total aflatoxin in the different divisions

The median aflatoxin concentrations across the four divisions were compared. The results showed that Lira City East Division had the lowest median concentration (1.128 ng/g), followed by Lira City West Division (1.492 ng/g). In contrast, Layibi-Bardege Division exhibited the highest median aflatoxin concentration (1.802 ng/g). Meanwhile, Pece-Laroo Division's median aflatoxin concentration (1.565 ng/g) fell intermediate to the other three divisions (figure 4.3). The error bar indicated no statistically significant difference in median aflatoxin concentrations between divisions at the 95% confidence level ($p > 0.05$).

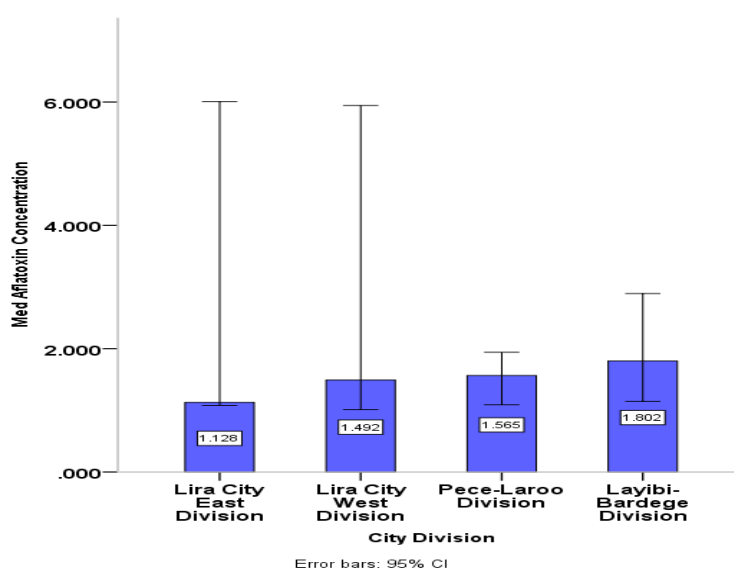


Figure 4. 3: A bar graph showing median aflatoxin concentration in the four division

Further, statistical analysis using the Kruskal-Wallis test ($H(3) = 0.944$, $p = 0.815$) revealed a p-value of 0.815, indicating that the median aflatoxin concentrations did not differ significantly among the four divisions ($p > 0.05$). In addition, a Mann-Whitney U test ($U = 32.000$, $Z = -0.711$, $p = 0.477$) was performed to compare the median aflatoxin concentrations between the two cities, and the results showed no significant difference ($p = 0.515$). These findings suggest that the aflatoxin concentrations were consistent across the different divisions and cities, with no significant variations detected.

4.1.3 Comparison of aflatoxin concentrations with regulatory standards

None of the herbal medicine samples analysed from the two cities exceeded the Ugandan regulatory limit of 10 $\mu\text{g/kg}$ for aflatoxin concentrations. The proportion of samples (11.1 %) with aflatoxin levels exceeding the European Pharmacopeia regulatory limit of 5 $\mu\text{g/kg}$ was significantly lower than those (88.9%) within the limit. A comparative analysis revealed that two samples from Lira City surpassed the European regulatory threshold, whereas no sample from Gulu City exceeded this limit (Table 4.2).

Table 4. 2: shows the levels of contamination found in this study and limits for Uganda and European Pharmacopeia

Sample ID	Aflatoxin concentration (ng/g)	Uganda National Drug Authority Regulatory limit (ng/g)	European Pharmacopeia regulatory limit (ng/g)
Lira City			
BL2	6.0058	10	5
CL1	1.1282	10	5
CL2	1.0782	10	5
DL2	1.6767	10	5
EL1	1.0111	10	5
EL3	5.9430	10	5
FL2	1.4923	10	5
FL3	1.1773	10	5
Gulu City			
GG4	1.0887	10	5
HG4	1.3620	10	5
IG2	1.7666	10	5
IG3	1.9424	10	5
JG2	2.1359	10	5
JG3	1.1596	10	5
KG1	1.1466	10	5
KG3	2.8941	10	5
KG4	1.8025	10	5
MG4	1.8025	10	5

Identification code for samples collected from herbal dealers in Gulu City (GG4, HG4, IG2, IG3, JG2, JG3, KG1, KG3, KG4, and MG4).

Identification code for samples collected from herbal dealers in Lira City (BL2, CL1, CL2, DL2, EL1, EL3, FL2, and FL3).

4.2 Health risk associated with consumption of herbal medicine

4.2.1 Estimated daily intake and Hazard index

The estimated daily intake for adults and children was calculated for total aflatoxin of herbal medicine consumed in Lira and Gulu Cities and presented in Table 4.3. The EDI ranged from 1.68×10^{-5} to 3.6×10^{-4} $\mu\text{g/kg.bw}$ for adults and 1.07×10^{-4} to 2.4×10^{-3} $\mu\text{g/kg.bw}$ for infants as shown in Table 4.3. The EDI in both cities was low compared to the tolerable daily intake of aflatoxin in herbal medicinal products. The recorded hazard index values ranged from 2.58×10^{-10} to 5.6×10^{-9} for adults and 1.65×10^{-9} to 3.6×10^{-8} for infants as shown in table 4.3. In general, it is accepted that an $\text{HI} \leq 1$ indicates no significant health risk. Although the Hazard Index (HI) values for total aflatoxin exposure in herbal medicines consumed in Lira and Gulu Cities were less than one, indicating a low risk, it is essential to note that increasing HI values

are associated with higher long-term adverse health effects. Specifically, HI values between 1.1 and 10 signify moderate risk, while values above 10 indicate high risk (Kortei *et al.*, 2019).

Table 4. 3: The calculated values of estimated daily intake and hazard index of aflatoxin in herbal medicine sold in northern Uganda

Samp le ID	Aflatoxin concentr ation (µ/kg)	Daily intake (g/day)	Daily intake (kg/day)	EDI (µg/kg. bw) for adults	EDI (µg/kg. bw) (infants)	HI for adults	HI for infants
BL2	6.0058	4.447	0.004447	0.0003694	0.0023635	5.6×10^{-09}	3.6×10^{-08}
CL1	1.1282	3.596	0.003596	0.0000561	0.0003590	8.6×10^{-10}	5.5×10^{-09}
CL2	1.0782	4.447	0.004447	0.0000663	0.0004243	1.0×10^{-09}	6.5×10^{-09}
DL2	1.6767	3.596	0.003596	0.0000834	0.0005336	1.2×10^{-09}	8.2×10^{-09}
EL1	1.0111	3.596	0.003596	0.0000503	0.0003218	7.7×10^{-10}	4.9×10^{-09}
EL3	5.9430	0.728	0.000728	0.0000598	0.0003829	9.2×10^{-10}	5.8×10^{-09}
FL2	1.4923	1.592	0.001592	0.0000329	0.0002102	5.0×10^{-10}	3.2×10^{-09}
FL3	1.1773	4.447	0.004447	0.0000724	0.0004633	1.1×10^{-09}	7.1×10^{-09}
GG4	1.0887	1.114	0.001114	0.0000168	0.0001073	2.5×10^{-10}	1.6×10^{-09}
HG4	1.3620	1.456	0.001456	0.0000274	0.0001755	4.2×10^{-10}	2.6×10^{-09}
IG2	1.7666	1.456	0.001456	0.0000356	0.0002276	5.4×10^{-10}	3.5×10^{-09}
IG3	1.9424	1.457	0.001457	0.0000391	0.0002505	6.0×10^{-10}	3.8×10^{-09}
JG2	2.1359	1.592	0.001592	0.0000470	0.0003009	7.2×10^{-10}	4.6×10^{-09}
JG3	1.1596	1.457	0.001457	0.0000234	0.0001495	3.5×10^{-10}	2.3×10^{-09}
KG1	1.1466	3.778	0.003778	0.0000599	0.0003833	9.2×10^{-10}	5.8×10^{-09}
KG3	2.8941	9.114	0.009114	0.0003648	0.0023342	5.6×10^{-09}	3.5×10^{-08}
KG4	1.8025	1.457	0.001457	0.0000363	0.0002324	5.5×10^{-10}	3.5×10^{-09}
MG4	1.8025	1.456	0.001456	0.0000363	0.0002322	5.5×10^{-10}	3.5×10^{-09}

TD50 of total Aflatoxin = 1.3 microgram per kilogram (µg/kg).

Identification code for samples collected from herbal dealers in Gulu City (GG4, HG4, IG2, IG3, JG2, JG3, KG1, KG3, KG4, and MG4).

Identification code for samples collected from herbal dealers in Lira City (BL2, CL1, CL2, DL2, EL1, EL3, FL2, and FL3).

4.3 Fungal identification

4.3.1 BLAST results

The sequence of the different fungal isolates was queried in the NCBI database and the results summarized in table 4.4. A total of 10 different fungal species were identified with the percentage identities ranging from 87.76 to 100.00

Table 4. 4: BLAST results obtained from NCBI Database.

Sample ID	Scientific Name	Max Score	Query Cover (%)	Per. Ident (%)	Accession Number
BL3A_ITS-1F	<i>Aspergillus flavus</i>	1066	98	99.83	MT529193.1
KG3_ITS-1F	<i>Aspergillus flavus</i>	1057	99	100.00	MN396681.1
CL2_ITS-1F	<i>Aspergillus flavus</i>	1062	99	99.83	OQ422083.1
BL2_ITS1F	<i>Aspergillus fllavus</i>	1020	98	97.59	MT645617.1
AL2_ITS1F	<i>Aspergillus fumigatus</i>	1074	99	99.32	MG552677.1
BL2A_ITS1-F	<i>Aspergillus fumigatus</i>	1085	99	100.00	MK431430.1
BL2B_ITS1F	<i>Aspergillus fumigatus</i>	1081	100	99.49	KX664348.1
FL2_ITS-1F	<i>Aspergillus terreus</i>	1085	98	99.83	MH237648.1
KG1_ITS1F	<i>Aspergillus terreus</i>	1098	99	99.83	MN860009.1
KG1A_ITS-1F	<i>Aspergillus terreus</i>	1083	100	99.33	MN860009.1
HG2_ITS1F	<i>Monascus ruber</i>	1033	100	98.96	MT316354.1
DL2_ITS1F	<i>Pichia kudriavzevii</i>	893	99	99.00	OK267906.1
EL2_ITS-1F	<i>Pichia kudriavzevii</i>	878	98	98.79	MG015974.1
EL-3_ITS1-F	<i>Pichia kudriavzevii</i>	885	99	98.60	KC905770.1
FL-2_ITS1-F	<i>Pichia kudriavzevii</i>	872	99	98.59	MG015972.1
IG2_ITS-1F	<i>Pichia kudriavzevii</i>	874	98	98.58	MH545928.1
IG3_ITS-1F	<i>Pichia kudriavzevii</i>	885	100	98.99	OR597934.1
KG1B_ITS1-F	<i>Pichia kudriavzevii</i>	880	97	98.59	KC905770.1
CL1_ITS1F	<i>Rhizopus arrhizus</i>	802	100	87.76	MN010549.1
FL-1_ITS1F	<i>Rhizopus microspores</i>	1258	100	99.42	MT279278.1

Sample ID for fungal species isolated from samples collected from Lira city (BL3A_ITS-1F, CL2_ITS-1F, BL2_ITS1F, AL2_ITS1F, BL2A_ITS1-F, BL2B_ITS1F, FL2_ITS-1F, DL2_ITS1F, EL2_ITS-1F, EL-3_ITS1-F, FL-2_ITS1-F, CL1_ITS1F, and FL-1_ITS1F)

Sample ID for fungal species isolated from samples collected from Gulu city (KG3_ITS-1F, KG1_ITS1F, KG1A_ITS-1F, HG2_ITS1F, IG2_ITS-1F, IG3_ITS-1F, and KG1B_ITS1-F)

4.3.2 Fungal species associated with herbal medicine in Lira and Gulu Cities

A total of 52 herbal medicine samples were collected and there was fungal growth in 18 samples (34.6%). 20 fungal isolates were amplified with internal transcribed spacer (ITS) primers and later sequenced. The nucleotide of the ITS region grouped the isolates into 7 different fungal species (Table 4.5). *Pichia kudriavzevii* was the most prevalent fungal species (N = 7, 35%). The second most prevalent fungal species in the herbal medicine from Lira and Gulu cities was *A. flavus* (N = 4, 20%). The proportion of isolates classified as *A. fumigatus* and *A. terreus* was 15%. Five percent (N=1) of the isolates were species: *Monascus ruber*, *Rhizopus arrhizus*, and *Rhizopus microsporus*. Three herbal medicine samples (KG1, FL2, and BL2) had fungal co-occurrence. Samples KG1 and FL2 showed co-occurrence of two fungal species: *Pichia kudriavzevii* and *Aspergillus terreus*. *Aspergillus flavus* and *Aspergillus fumigatus* were identified as co-occurring fungal species in sample BL2. This implies that those herbal medicines having fungal co-occurrence may be contaminated with multiple classes of aflatoxin.

Lira City had the highest number of fungal isolates (12) and Gulu had 8 (Table 4.5). The isolate with the highest proportion was *Pichia kudriavzevii* in both cities. *A. terreus*, *Monascus ruber* species were identified in Gulu City only. In addition, species *Rhizopus arrhizus* and *Rhizopus microsporus* were only identified in Lira (Table 4.5).

Table 4. 5: Fungal species isolated from Herbal Medicine in Lira and Gulu Cities (n = 20)

Species classification based on ITS region		N	Percentage (%)
	<i>Aspergillus flavus</i>	4	20
	<i>Aspergillus fumigatus</i>	3	15
	<i>Aspergillus terreus</i>	3	15
	<i>Monascus ruber</i>	1	5
	<i>Pichia kudriavzevii</i>	7	35
	<i>Rhizopus arrhizus</i>	1	5
	<i>Rhizopus microspores</i>	1	5
	Total	20	100
City			
Gulu	<i>Aspergillus flavus</i>	1	12.5
	<i>Aspergillus terreus</i>	3	37.5
	<i>Monascus ruber</i>	1	12.5
	<i>Pichia kudriavzevii</i>	3	37.5
	Total	8	100
Lira	<i>Aspergillus flavus</i>	3	25
	<i>Aspergillus fumigatus</i>	3	25
	<i>Pichia kudriavzevii</i>	4	34
	<i>Rhizopus arrhizus</i>	1	8
	<i>Rhizopus microspores</i>	1	8
	Total	12	100

4.3.3 Phylogenetic tree of the *Aspergillus* and genus identified

A phylogenetic tree (figure 4.4) was inferred from ITS gene sequence and showed that the three different *Aspergillus* species (*A. flavus*, *A. terreus*, and *A. fumigatus*) diverged to form three sub-clades. Amongst the three *Aspergillus* species, *A. flavus* formed a further distant sub-clade. *A. fumigatus* and *A. terreus* were more closely clustered.

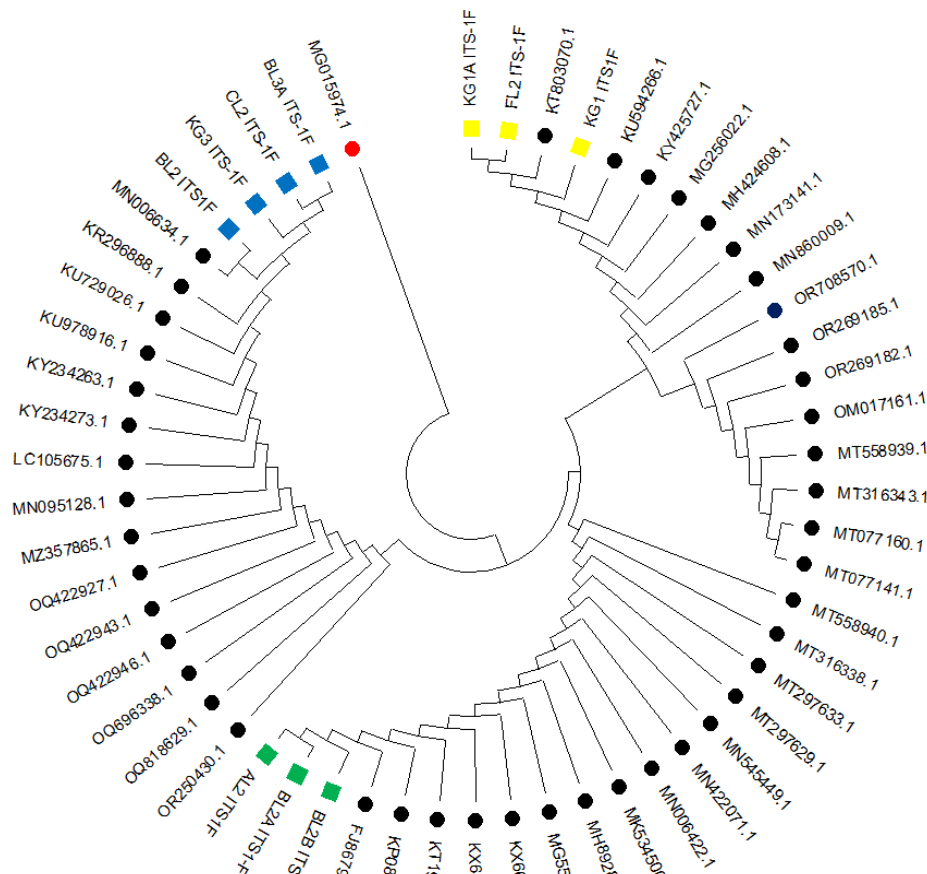


Figure 4. 4: The phylogenetic tree of the *Aspergillus* genus identified

Phylogenetic tree showing cluster separation among *A. flavus*, *A. terreus*, and *A. fumigatus*. The green, yellow, and blue colour represent *A. fumigatus*, *A. terreus*, and *A. flavus*, respectively. The black colour represents the reference isolates and red colour shows the outgroup isolate.

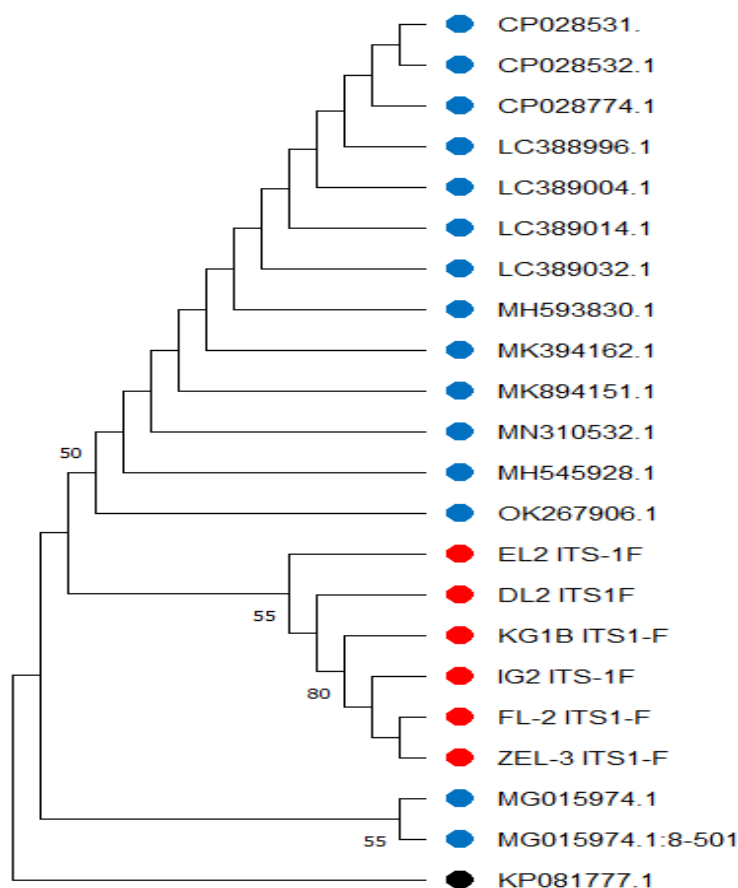


Figure 4. 5: The phylogenetic tree of the *Pichia kudriavzevii* species identified

The sample isolate of *Pichia kudriavzevii* (indicated with a red colour) clustered together. The black colour represents the outgroup isolate and blue shows the reference isolates. The clustering indicates shared ancestry among sequences, revealing evolutionary relationship.

4.4 Correlation between aflatoxin concentration and herbal moisture content

The statistical output indicates a moderate positive correlation between aflatoxin concentration and moisture content in the herbal medicines as shown in Table 4.6. This means that as the moisture content increases, the level of aflatoxin contamination also increases. This finding is statistically significant, ($df = 16$; $r = 0.51$; $p = 0.028$), suggesting that the observed relationship is unlikely due to chance.

Table 4.6: Spearman rank correlation between aflatoxin concentration and herbal moisture content

Correlations				
			Aflatoxin Concentration	Moisture content
Spearman's rho	Aflatoxin Concentration	Correlation Coefficient	1.000	.517*
		Sig. (2-tailed)	.	.028
		N	18	18
	Moisture content	Correlation Coefficient	.517*	1.000
		Sig. (2-tailed)	.028	.
		N	18	18

*, Correlation is significant at the 0.05 level (2-tailed).

CHAPTER FIVE: DISCUSSION

The quality of herbal medicine can be compromised by aflatoxin contamination during processing and storage. This poses a health threat to herbal medicine consumers worldwide. Numerous natural occurrences of aflatoxin in medicinal plants and traditional herbal medicines have been reported in various countries.

This study examined the level of aflatoxin and fungal contamination in herbal medicinal products sold in Gulu and Lira cities. The analysis of total aflatoxin concentrations indicated that 35% of the herbal samples were contaminated. The findings correspond to a study done by Jallow *et al.* (2021), which indicated that 35% of the herbal medicine samples were contaminated with aflatoxin. However, Ab Dullah *et al.* (2023) and Wei *et al.* (2023) found proportions of herbal medicine contaminated with aflatoxin at 80.6 and 62.92%, respectively, which was much higher than the levels reported in this study. Conversely, studies conducted by Ezekwesili-Ofili *et al.* (2014) and Tassaneeyakul *et al.* (2004) reported aflatoxin levels of 18% and 18.6%, respectively, both of which were lower than our findings. The occurrence of aflatoxin is indicative of contamination by aflatoxigenic fungi in the herbal medicinal product. This is mainly due to herbal dealers' failure to employ contemporary/modern processing methods, resulting in poor/inadequate and unhygienic management of herbal products. This subsequently results in fungal contamination and aflatoxin production. The incidence of aflatoxin also implies that herbal medicinal products are susceptible to infection by aflatoxigenic fungi. This also reveals that the raw materials (plant parts) used for manufacturing herbal medicines lack chemicals that inhibit fungal growth. According to Singh *et al.* (2008), essential oil of *Cinnamomum camphora* has been reported to suppress fungal growth and the production of AFB1 in raw materials used to make herbal medications. Essential oils from *Ageratum conyzoides* L Patil *et al.* (2010) and *Satureja hortensis* L. (Razzaghi-Abyaneh *et al.*, 2008) have demonstrated efficacy in inhibiting the growth of *A. parasiticus* and the production of aflatoxin. Similarly, extracts of *Garcinia cowa* and *G. pedunculata* inhibit the growth and synthesis of aflatoxin by *A. flavus* (Joseph *et al.*, 2005).

The present study investigated the fungal diversity associated with herbal medicinal products sold in Gulu and Lira cities, in northern Uganda. A total of 20 fungal isolates were identified from 18 samples, with the *Aspergillus* genus constituting 50%. The similarity in contamination levels between the two cities suggests that the factors contributing to fungal and aflatoxin contamination, such as poor agricultural practices, sub-optimal storage

conditions, and processing methods, are similar. In the same accord, the two cities exhibit analogous climatic conditions. In northern Uganda, the high humidity and rainfall, coupled with high temperatures, especially during the two annual rainy seasons, often affect the proper drying of herbal medicine before storage. The inadequately dried raw materials (plant parts) are associated with fungal growth and aflatoxin contamination, mainly due to the high moisture content (Chandra *et al.*, 2019). This is corroborated by the high moisture content levels found in the majority of the herbal medicinal products analysed during the study. Fungal growth during storage is further exacerbated by poor aeration and favourable temperature. This problem of poor storage would be alleviated if herbal medicine manufacturers adopted modern drying technologies like microwave drying, oven drying, and solar drying as this would help reduce the moisture content level. This underscores the necessity for improved manufacturing practices. Further lessening of fungal and aflatoxin contamination would be achieved by proper packaging, transportation, and stringent monitoring of the safety and efficacy of herbal medicine.

The findings of this study revealed no substantial disparity in aflatoxin contamination between Gulu and Lira Cities, implying that both cities encounter similar challenges in managing aflatoxin contamination in herbal medicines. To alleviate aflatoxin contamination, appropriate measures such as the "antagonistic storage" method based on the "couplet medicine" method, should be implemented during the storing of herbal medicines (Ying *et al.*, 2018). This method facilitates the co-storage of one herbal medicinal product (protector) that has a strong anti-fungal property, alongside another herbal medicinal product (protege), which is susceptible to various fungal proliferation, thereby inhibiting fungal growth and aflatoxin production in the protege for a long period of safe co-storage. The technique is easy to operate, leaving no residue of other ectogenic toxic components and is environmentally sustainable (Wei *et al.*, 2023; Ying *et al.*, 2018). This would serve as a prospective eco-friendly solution to greatly reduce the aflatoxin contamination.

A comparison with regulatory limits revealed that none of the samples exceeded Uganda National Drug Authority regulatory limit of 10µg/kg, however 11.1% exceeded the European Pharmacopoeia limit of 5µg/kg. The detection of aflatoxin concentrations exceeding the European Pharmacopoeia threshold underscores the necessity for implementing more stringent regulations to guarantee the safety of herbal medicines in northern Uganda. The increasing consumption of contaminated herbal medicinal products has made their use a public health problem due to the lack of effective surveillance of their utilisation, efficacy,

toxicity, and quality. The increase in the use of medicinal plants may lead to an increase in the intake of aflatoxins; therefore, contamination of medicinal plants with aflatoxins can contribute to adverse human health problems and therefore represents a special hazard (Javed *et al.*, 2021). Given the widespread use of herbal medicines in northern Uganda as reported by Nyeko *et al.* (2016), the findings of our study are appalling, considering that a significant portion of the population in this region depends on herbal medicine for health issues. This implies that many people in northern Uganda are at a risk of the deleterious effects of aflatoxin.

A health risk assessment using Hazard Index (HI) values indicated that the intake of herbal medicines in Gulu and Lira cities is unlikely to pose a significant health risk to the local population in the short term. However, regular consumption over an extended period may lead to long-term effects, including liver damage and increased risk of chronic diseases (Onen *et al.*, 2021). This validates the need for continued monitoring and mitigation strategies to minimize exposure.

Phylogenetic analysis of fungal isolates from herbal medicine samples revealed a predominance of the *Aspergillus* genus, with distinct sub-clades reflecting evolutionary relationships. *A. flavus*, a known aflatoxin producer, formed a separate subclade, while *A. fumigatus* and *A. terreus* were more closely clustered. This information is valuable for understanding the genetic diversity and potential pathogenicity of these fungi.

The prevalence of fungal contamination in herbal medicine can be attributed to the high moisture content in these products. The moisture content analysis showed that 32.7% of samples exceeded permissible limits, providing an ideal environment for *Aspergillus* growth and aflatoxin production. The moisture content of herbal medicines is a critical factor influencing aflatoxin contamination. High moisture levels can facilitate fungal growth, while low moisture levels can suppress it (Kumar *et al.*, 2021). A notable positive association between aflatoxin concentration and moisture content emphasizes the necessity of regulating moisture levels in herbal medicinal products to mitigate contamination risk.

CHAPTER SIX: CONCLUSION

This study investigated the prevalence of aflatoxin and fungal contamination in herbal medicinal products sold in Gulu and Lira cities, northern Uganda. A significant proportion (35%) of samples was contaminated with aflatoxin, with 11.1% exceeding international regulatory tolerable limits. The presence of aflatoxin indicates the contamination of herbal medicines with aflatoxigenic fungi, likely due to poor processing and storage practices.

The study identified a diverse range of fungal species, with *Aspergillus* genus being the most prevalent. High moisture content in herbal medicine samples was found to be a key factor contributing to fungal growth and aflatoxin production. The correlation between moisture content and aflatoxin concentration underscores the importance of proper drying and storage techniques to mitigate contamination risks.

While the health risk posed by aflatoxin exposure through herbal medicine consumption in the short term appears to be low, regular consumption over an extended period may lead to long-term health effects. Therefore, it is crucial to implement effective measures to reduce aflatoxin contamination in herbal medicines.

In conclusion, aflatoxin contamination in herbal medicines poses a significant public health risk, necessitating urgent attention and action. By adopting a multifaceted approach to mitigate contamination, including improved agricultural practices, enhanced processing and quality control measures, and stricter regulatory standards, we can ensure the safety and efficacy of herbal medicines for consumers.

6.1 Recommendations

To tackle the issue of aflatoxin contamination in herbal medicines, a comprehensive approach is crucial. Processing and quality control measures should be put in place by Uganda National Drug Authority, including proper drying, storage, and packaging techniques to prevent moisture build-up and fungal growth. This is particularly important in northern Uganda, where high humidity and rainfall can exacerbate contamination.

Stricter regulatory standards are also essential to enforce regulations that guarantee the safety and quality of herbal medicines. This can involve regular inspections, testing, and certification of herbal products by National Drug Authority to ensure compliance with established standards.

Raising awareness among herbal manufacturers to reduce the moisture content of herbal medicine, as it is one of the factors that enhance microbial activities and subsequent aflatoxin production. Raising awareness among consumers is also vital, and public awareness campaigns can educate people about the risks associated with aflatoxin contamination and the importance of selecting safe herbal products. By empowering consumers with knowledge, they can make informed decisions about their health. A future research is needed to understand the long-term consequence of exposure to low levels of aflatoxins.

Finally, exploring innovative storage methods, such as "antagonistic storage," can help reduce fungal growth and aflatoxin production. This eco-friendly approach involves storing herbal medicines with plants that have natural anti-fungal properties, creating a protective environment that prevents contamination. A promising area for future research is to explore the potential use of natural antifungal agents, such as essential oils or plant extract, to control fungal growth and aflatoxin production in herbal medicine.

By adopting these strategies, we can significantly reduce the risk of aflatoxin contamination in herbal medicines and ensure their safety for consumers in northern Uganda. This multifaceted approach requires collaboration among farmers, manufacturers, regulators, and consumers to create a safe and healthy herbal medicine industry.

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Appendices

Appendix I: Sample Collection Form (version 2.0)

Date of collection.....

Name of herbal dealer.....

Village

Division.....

City.....

GPS Coordinates.....

Sample identity	Herbal dealer

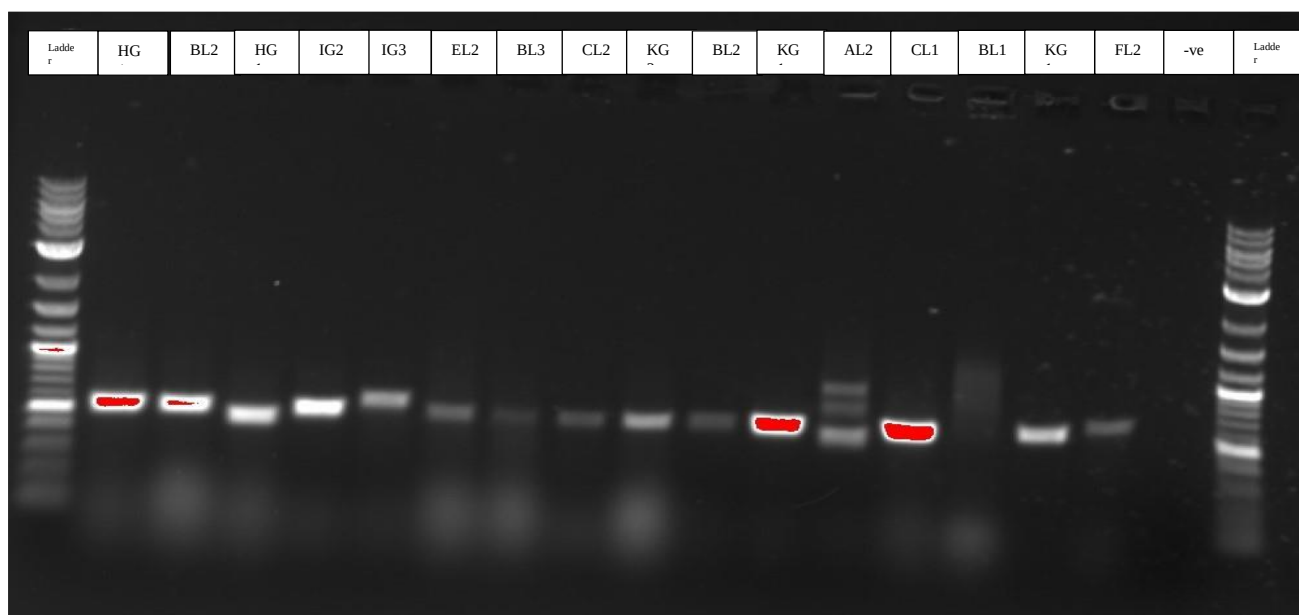
Appendix II: Fungal species isolated from the herbal medicine (samples) (version 2.0)

Sample ID	Source (herbal dealer)	Number of fungal species

Appendix III: Data collection sheet for determining aflatoxin (version 2.0)

Sample ID	Replication ID	Concentration of aflatoxin	Mean level of aflatoxin

Appendix IV: Amplification of the fungal ITS region



Appendix V: Plagiarism Check

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