Aflatoxin content and health risks associated with consumption of some herbal products sold in Kampala, Uganda

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Persuasive adverts and exaggeration of health benefits from consumption of herbal products as well as the mental picture of 'natural' is 'safe' has boosted traditional medicine use in Uganda. However, herbal products may be unsafe due to the possibility of their contamination with mycotoxins. In this study, we quantified the levels of aflatoxins (B_1, B_2, G_1 and G_2) in Real Koff product, *Eddagala ly'e kifuba n'e senyiga*, *Omusujja*, Cough mixture and Fever herbal products sold in Kampala, Uganda using high performance liquid chromatography-tandem mass spectrometry. The associated consumption health risks were assessed using the hazard index method. Only aflatoxin B_1 was detected in 60% of the samples, with 40% of these surpassing WHO guidelines of ≤ 5 µg/kg. The hazard indices were all less than 1, implying that Ugandans who heavily rely on the herbal products are exposed to aflatoxins at sublethal doses which may lead to chronic effects in the long run. Studies using a larger sample size should assess whether the current observation is a routine occurrence or a sporadic event.

Introduction

Plants have been known for their medicinal potential since antiquity [1]. They continue to be a veritable source of not less than 40% of the current therapeutically utilized products. Some studies have indicated that the use of herbal products to address health challenges could be unsafe due to the possibility of their contamination with heavy metals [2, 3], pesticide residues, and toxigenic fungi such as *Aspergillus*, *Fusarium* and *Penicillium* species during processing and packaging [4, 5]. These fungi are

known to produce potential mycotoxins (aflatoxins, fumonisins, ochratoxins, deoxynivalenol, zearalenone and T-2 toxins), which may have negative health impacts on humans [6].

Aflatoxins (AFs) is a group of secondary metabolites produced by fungi of the genus *Aspergillus*, majorly *A. flavus* and *A. parasiticus*. About 20 different types of AFs have been recognized. However, aflatoxin B_1 (AFB_1), aflatoxin B_2 (AFB_2), aflatoxin G_1 (AFG_1), aflatoxin G_2 (AFG_2), aflatoxin M_1 (AFM_1) and aflatoxin M_2 (AFM_2) are of utmost toxicological importance [7]. AFs have a wide occurrence in different matrices. They have been reported in agricultural foods including cereals (rice, maize, sorghum, millet), cassava, nuts, oil seeds (such as sesame, sunflower), meat, eggs, dairy products, spices and animal feeds [8]. In foods and herbal products, they may occur before, during or after harvest, during storage or processing under conditions that favour the survival of the producing *Aspergillus* fungi [9-11].

In our previous study [6], we concluded that various commodities in Uganda are contaminated with AFs, and most communities are exposed to acute chronic levels. Furthermore, we identified that herbal products, beers, barley and animal products (meat and blood) have been barely assessed in regard to these toxins. The aim of this study was therefore to quantify aflatoxins (AFB_1, AFB_2, AFG_1 and AFG_2) in Real Koff, *Eddagala ly'e kifuba n'e senyiga*, *Omusujja*, Cough mixture and Fever herbal products traded in Kampala city of Uganda and assess the probable human health risks from their consumption.

Experimental part

Materials and methods

Aflatoxin (AFB_1, AFG_1, AFB_2 and AFG_2) standards were procured from Sigma (St. Louis, MO, USA). Ethanol, methanol, and acetonitrile were from Sigma (Sigma-Aldrich, Steinheim, Germany). All other reagents used were of analytical grade, supplied by Merck (Darmstadt, Germany) and Sigma (St. Louis, MO, USA).

Sample collection and preparation

Liquid dosage forms of five common herbal medicines in their original packaging were each obtained from 15 popular herbal shops in Kampala, Uganda on 15th December 2019 (**Table 1; Figure 1**). Kampala was chosen because of the big market and high sale turnover of herbal medicines [12]. Selection of these herbal formulations was also based on their availability and affordability by the public following a pilot survey. Samples were analyzed at Directorate of Government Analytical Laboratory, Wandegeya, Kampala, Uganda.

Table 1. Liquid herbal medicines sampled from Kampala, Uganda.

ID	Trade name	Ailment(s) claimed to treat
SL1	Real Koff product	Cough and flu
SL2	*Eddagala ly'e kifuba n'e senyiga*	Cough and flu
SL3	*Omusujja*	Various ailments
SL4	Cough mixture	Cough, asthma, fever, ulcers, measles
SL5	Fever	Fever

Figure 1. One set of the sampled herbal medicines in their original packaging (left to right: Real Koff product, *Eddagala ly'e kifuba n'e senyiga*, *Omusujja,* Cough mixture and Fever).

Aflatoxin analysis

The working standards for AFs: 125 µL for AFB_1 and AFG_1, 450 µL for AFB_2 and AFG_2 were diluted to 5 mL with acetonitrile/water (50: 50 v/v) resulting in an intermediate mixed solution of 0.05 µg/mL for AFB_1 and AFG_1, 0.047 µg/mL and 0.045 µg/mL for AFB_2 and AFG_2, respectively.

Aliquots (30 ml) of each sample were extracted using 10 ml acetonitrile-water (84: 16 v/v) for 30 minutes with shaking at room temperature at 126 rpm. They were then centrifuged for 5 minutes at 3000 × g. Solid phase extraction was done and 0.4 ml of the extracts were diluted with 0.6 ml of ammonium formate in water.

Determination of aflatoxin levels in the sample extracts was performed by isocratic reversed-phase high performance liquid chromatography-tandem mass spectrometry with a MS/MS detector from Agilent Technologies (6400 Series Triple Quadrupole B.08.00, B8023.0). The analyses used binary gradient elution with solvent A (a mixture of 0.1% formic acid in water and 0.1% ammonium formate in water) while solvent B was made of 0.1% formic acid in methanol [13]. The total run time was 7 minutes.

Sample solutions of 5 µL were injected into C-18 reverse phase column (Poroshell 120 EC-C18 3 × 50 mm, 2.7 µm, USA). Data acquisition software was for 6400 Series Triple Quadrupole, version B.08.00 while qualitative analysis software used was version B.07.00 Service Pack 1. The flow rate was set at 0.3 mL/min and the column temperature maintained at 40.01 ºC. The ionization source of the MS/MS detector had 4.0 kV capillary voltage, 350 ºC source temperature and 10 mL/min desolvation gas flow using nitrogen gas (Supplementary File 1. Table S1)[13].

The concentration of each aflatoxin in the samples were separately calculated by using the individual calibration curves obtained from the peak heights of each aflatoxin standard. Standards and samples were analyzed with a method detection limit of 0.03 µg/kg.

Quality control was achieved through triplicate analysis of samples.

Human health risk assessment

The estimated daily intake (EDI) for ingestion of the herbal medicines as well as the hazard index (HI) were computed for both children (representing a sensitive group) and adults (representing the general population) to

approximate probable exposure through intake of herbal medicines. This was because traders reiterated that most people believed in the herbal products and take them daily, some along with their children, as a measure of preventing the ailments.

The EDI was calculated using the mean levels of total aflatoxins obtained in the herbal medicines, the daily herbal intake of 0.25 L/day and 0.75 L/day for Ugandan children and adults respectively [14] and the mean body weights of 11.3 kg and 72.3 kg/person for children and adults respectively [15-17]. The EDI expressed in ng/kg of the body-weight/day (ng/kg bw/day) was calculated using **Equation 1** [17-19].

$$\text{EDI} = \frac{\text{Daily intake of herbal medicine} \times \text{Mean level of aflatoxins}}{\text{Average body weight in Kg}} \quad (1)$$

Where daily intake of herbal medicine is in L/day, mean level of aflatoxins in ng/kg and average body weight in kg.

The hazard index was determined by dividing the EDI by TD_{50} (the daily dose in ng/kg/bw/day at which 50% of test animals would have developed tumours), divided by a safety factor of 50,000 as described by previous authors [17, 19-21]. TD_{50} is the dose (mg/kg/bw/day) required to induce tumors in half of the test animals that would have remained tumor-free at zero dose. The complete mathematical relation of these parameters is given in **Equation 2**.

$$\text{HI} = \sum_{n=0}^{i} \frac{\text{EDI}/\text{TD50}}{50{,}000} \quad (2)$$

Statistical analysis

All quantitative data, unless otherwise stated, were presented as means with errors represented by standard deviations attached. The analyses were done using Minitab statistical software (v17, Minitab Inc., USA). The aflatoxin content of the samples was compared with the acceptable levels of aflatoxins in herbal products recommended by World Health Organization, European and United States pharmacopeias.

Results and Discussion

Aflatoxin content of the herbal medicines

The current study evaluated the aflatoxin content of some herbal products sold in Kampala (Uganda) using a combination of high-performance liquid chromatography and liquid chromatography-tandem quadrupole mass spectrometry. Despite the existence of several methods described for the determination of AFs in matrices [7], none of them can be applied to all matrices. In the case of natural products, the difficulty stems from the chemical complexity of different compounds in medicinal plants [22]. However, high-performance liquid chromatography-tandem quadrupole mass spectrometry (HPLC-MS/MS) has been reported and validated to determine the presence of AFs more accurately than HPLC and avoids the tedious derivatization process [23]. This method overcomes the drawbacks of HPLC by using tandem MS/MS detection, and is regarded as a confirmatory method for quick determination of AFB_1, AFB_2, AFG_1 and AFG_2 [23]. In this study,

we did not revalidate this method as it was already validated in-house by the same laboratory which handles at least 50% of samples requiring AFs analyses from most parts of the country.

The aflatoxin content of the herbal products are given in **Table 2**. AFB_1 was detected in 60% (3/5) of the herbal products: Real Koff product, *Omusujja* and Fever with mean AFB_1 levels of 6.83 ± 0.04, 6.38 ± 0.03 and 4.97 ± 0.07 µg/kg, respectively. Real Koff product and *Omusujja* had AFB_1 in levels above WHO guideline, European pharmacopoeia as well as United States pharmacopoeia of ≤5, ≤2 and ≤5 µg/kg, respectively. In a previous study, Odda et al. [11] reported that the shoot powder of *Phyllanthus amarus* (Schum. and Thonn) from Tororo (Uganda) were contaminated with AFs in levels above the recommended limits for total AFs by WHO. In the current study, it was found that among the four AFs, AFB_1 was detected as the most common and only contaminant. This is in consonance with previous reports [22-24] in which AFB_1 was the dominant contaminant. This is usually because *A. flavus* which produces AFB_1 and AFB_2 is a very common fungus which infect plants. Conversely, AFG_1 and AFG_2 are less often detected in plant products because the producing fungus (*A. parasiticus*) does not infect plants very often [23, 25, 26]. This may account for the high incidence and concentrations of AFB_1 compared with AFG_1 and AFG_2 in the herbal products investigated in this study. In addition, the total absence of other AFs in herbal products have been reported before [23, 27, 28].

Table 2. Aflatoxin content of the sampled herbal medicines from Kampala, Uganda.

Sample ID	Aflatoxin concentration (µg/kg)				
	AFB_1	AFB_2	AFG_1	AFG_2	Total AFs
SL1	6.83 ± 0.04	BDL	BDL	BDL	6.83
SL2	BDL*	BDL	BDL	BDL	NA
SL3	6.38 ± 0.03	BDL	BDL	BDL	6.38
SL4	BDL	BDL	BDL	BDL	NA
SL5	4.97 ± 0.07	BDL	BDL	BDL	4.97
WHO guideline	≤5	NE	NE	NE	NE
European pharmacopeia	≤ 2	NE	NE	NE	NE
US pharmacopeia	≤5	NE	NE	NE	NE

*BDL: Below method detection limit, **NA: Not applicable, NE: No established limit.

A plausible explanation for this observation could be due to the absence of the toxigenic fungi in the samples, the phytochemicals present in the products [29, 30] or environmental conditions during harvest and storage that did not favour biosynthesis of the other AFs. It is documented that temperature and water activity are the main factors that influence fungal invasion and the production of AFs in stored products. For example, Kulshrestha et al. [31] earlier reported that with a water activity below 0.81, temperatures of 25 ± 2 ℃, 30 ± 2 ℃, and 40 ± 2 ℃, and water activity over 0.81 and temperature below 10 ± 2 ℃, AFs were not detected in medicinal products that contained *A. flavus* (an aflatoxin producer). Similar results were later reported in Italy with no AFs detected in 48 infusions and medicinal plants analyzed [28].

Though 40% and 10% of the samples had undetectable and allowable aflatoxin levels in herbal products, it should be noted that low level intake of AFs over long periods result in chronic aflatoxicosis expressed as impaired food conversion, stunting in children, immunosuppression, cancer and reduced life expectancy [32-35].

Human exposure assessment

The estimated daily intakes of the herbal medicines ranged from 5.61×10^{-2} µg/kg/bw/day to 1.51×10^{-1} µg/kg/bw/day for both Ugandan children and adults (**Table 3**).

Table 3. Human health risk assessment for Ugandans via intake of the herbal medicines.

Index	Sample	Children	Adults
EDI (µg/kg/bw/day)	SL1	1.51×10^{-1}	7.09×10^{-2}
	SL2	Not applicable	Not applicable
	SL3	1.41×10^{-2}	6.62×10^{-2}
	SL4	Not applicable	Not applicable
	SL5	1.09×10^{-1}	5.16×10^{-2}
Hazard Index	SL1	2.32×10^{-6}	1.09×10^{-6}
	SL2	Not applicable	Not applicable
	SL3	2.17×10^{-6}	1.02×10^{-6}
	SL4	Not applicable	Not applicable
	SL5	1.69×10^{-6}	7.93×10^{-7}

The recorded hazard indices were in the range of 7.93×10^{-7} to 2.32×10^{-6}. Hazard indices less than or equal to 1 indicates no significant health risk but the possibility of long-term adverse health effects increases with increasing hazard index. Hazard index between 1.1 and 10 reflects a moderate risk while hazard index greater than 10 indicates high risk [17]. The computed hazard indices were all less than 1, implying that daily consumption of the individual herbal products may not pose health risks. However, the risk is higher in children. This is due to their higher dietary exposure per kg body weight and the differences in their body physiology when compared to adults [36]. The risks may also be amplified if two or more of the herbal products are consumed in tandem.

Conclusions

In the current study, we found that three out of the five commonly used herbal medicines in Kampala, Uganda were contaminated with aflatoxin B_1, a potent carcinogen. Thus, Ugandans who heavily rely on herbal products could be exposed to aflatoxins at sublethal doses, which could have long term carcinogenic effects. We recommend further studies using a large sample size and in other parts of Uganda. The mycoflora associated with the contamination as well as co-ocurrence of aflatoxins with other mycotoxins in Ugandan herbal products should be investigated.

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