

Evaluation of aflatoxins B1, B2, G1 and G2 traditional and industrial barley malts in Ethiopia

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Abstract

Background: The commonly used ingredient in home-made and conventional beer brewing is the traditional and industrial barley malt. However, this cereal grain is prone to pre-harvest and post-harvest contamination with aflatoxin. Aflatoxins have been shown in many studies to be teratogenic, mutagenic, genotoxic and hepatocarcinogenic to humans and animals.

Objective: This research was conducted to evaluate the presence of aflatoxin B1 (AFB1), B2 (AFB2), G1 (AFG1) and G2 (AFG2) in both traditional and industrial barley malts in Ethiopia.

Methods: Samples of 24 traditional barley malts (8 samples from each towns) were collected from Debre markos, Finoteselam and Enjibarra towns. Six industrial barley samples (3 local and 3 imported) were collected from three breweries. Aflatoxins B1, B2, G1 and G2 were determined by immunoaffinity column clean up and high performance liquid chromatography with fluorescence detection.

Results: The mean aflatoxin level of traditional barley malts from Debre markos, Finoteselam and Enjibarra towns were 5.30 µg/kg, 1.90 µg/kg and 6.72 µg/kg, respectively. The percentages of barley malt samples contaminated by aflatoxins are 37.5% in both Debre markos and Finoteselam and 50% in Enjibarra. The mean moisture content of the barley malt samples were 6.6%, 9.7% and 7.4% for Debre markos, Finoteselam and Enjibarra, respectively. Mean aflatoxin content of traditional malts from Debre markos and Enjibarra exceed the regulation of the European Commission. The aflatoxin content of imported malts exceeds those of industrially produced local malts. The aflatoxin content of industrially produced local malts are below the permitted limits of EU regulation in contrast to imported malts. In conclusion, the present study provides information about the occurrence of aflatoxins in traditional and industrial barley malts. The results of the study revealed that, consumption of both home-made and conventional beer may pose a risk of aflatoxin exposure.

Keywords: Aflatoxin, HPLC, industrial barley malt, traditional barley malt.

Introduction

Barley (*Hordeum vulgare* L.) is one of the most important cereal crops that largely produced in the central and south east mid and high altitude areas of Ethiopia. It is the fifth important cereal crop after tef, maize, wheat and sorghum with annual production of 1.9 million tons in 2014 (CSA 2014). Ethiopia is the second largest barley producer in Africa next to Morocco accounting for about 25 % of the total barley production in the continent (FAO 2009). Barley is the primary cereal widely used in the preparation of malt for brewing beer and other distilled liquors. This was perhaps due to its ability to produce α -amylase and β -amylase that take part in hydrolyzing starch, but also partly due to its morphology (tightly cemented lemma and palea which protects embryo and results in uniform germination) (Burger and Laberge 1985).

Brewing and consumption of traditional beer have socio-economic significance in most African countries including Ethiopia. Barley is one of the staple food crops used to prepare *injera*, porridge, bread and snacks and local drinks (*Tella* and *Besso*). Traditional barley malt is a starting material for the production of

the traditional alcoholic beverage, *Arakie*. Cereal crops such as barley, sorghum, maize, wheat and corn are susceptible for mycotoxin contamination. Mycotoxins are naturally occurring secondary metabolites of fungi and toxic to humans, animals and plants. *Aspergillus*, *Fusarium*, and *Penicillium* species are the most significant fungal pathogens attacking agricultural commodities. Grain contamination and damage may occur in the field during kernel maturation, harvest, transportation and storage processes (Volkova 2013). Aflatoxins are a group of naturally-occurring, structurally related polyketide mycotoxins and are produced by many species of *Aspergillus* fungi most notably *Aspergillus flavus* and *Aspergillus parasiticus*. Types of aflatoxins are AFB1, AFB2, AFG1, AFG2 and the AFM1 and AFM2 are after metabolizing of AFB1 and AFB2 respectively. These mycotoxins contaminate many agricultural commodities, such as corn, barley wheat, legumes, nuts spices and condiments (Habtamu and Kelbessa 2001). Milk and milk products can be contaminated as a result of cows being fed on aflatoxin-contaminated feed (Bhat et al. 2010).

Aflatoxin production is the consequence of a combination of species, substrate and environment: in storage conditions at temperature of 20-40°C, 10-20% of moisture and 70-90% of relative humidity of the air (Volkova 2013). Aflatoxins are produced at the end of the exponential phase or at the beginning of the stationary phase of the mould growth.

Malt is the main brewing grain material prepared from barley by germination and modification processes, whereas malting is the process of converting barley or other cereal grains into malt for use in brewing, distilling or in foods and called a malt house or a malting floor (Volkova, 2013). Malt is used to supply fermentable sugars, amino acids and vitamins required for growth and alcoholic fermentation (Hattingh 2013). Barley malt has a major influence on the organoleptic quality of the final product. Sugars, amino acids and lipids contribute to colour and flavor compounds, while proteins and polysaccharides are for foam formation and the body of beer (Ullrich 2011).

In Ethiopia, the potential areas for supplying malt barley for both traditional and industrial malting are Arsi and Bale in Oromiya Region as well as Gojam and Gonder in Amhara Region (Mulatu and Grando 2011). There are two malt plants in Ethiopia, Assela malt factory and Gonder malt factory. Traditional malt is produced in Ethiopia by individual households by soaking cleaned cereals (wheat, barley or sorghum) in water for 3-4 days, draining soak water, spreading on flat plates, covering with leaves of *ensete* or *Vernonia amygdalina* and left to germinate for 3-4 days. The germinated cereals are then spread on plastic mats and sun dried. Barley malt is essential to the beverage industry as it is the predominant raw material in beer production worldwide (Ross et al. 2002).

The industrial production of usually consists of four stages: grain cleaning and grading, steeping, germination and kilning. Each stage in the process is carefully monitored to ensure good malt quality (Hattingh 2013). Barley germination, has high moisture and temperature conditions which are favorable for yeast and mould growth; and the production and accumulation of extra amounts of mycotoxins mostly *Aspergillus* and *Fusarium* toxins may occur (Fukal et al. 1990). Although fungal and mycotoxin contamination of barley grains has been reported extensively, no studies have been carried out to determine the mycotoxicological risk that barley malts in Ethiopia represent for local and industrial beer consumption. This work was aimed to quantify aflatoxins in traditional and industrial barley malts produced in Ethiopia due to this information scarcity.

Materials and Methods

The study was conducted in Debreworkos, Finoteselam, Enjibarra, Gonder, and Addis Ababa. Samples of traditional barley malts were collected from Debreworkos, Finoteselam and Enjibarra in Amhara Regional State, Ethiopia. These towns are best known for their traditional beer (*Tella*) brewing and consumption. A total of 24 samples (eight samples from each town) were collected. Samples (3 kg each) were collected randomly from the markets of these towns, homogenized and divided to obtain a 1 kg laboratory sample for analysis. From each three different breweries, one locally produced and one imported malt samples were collected. Barley malt samples were blended, homogenized and divided to obtain a 1 kg laboratory sample. All samples were analyzed immediately for fungi, then milled and stored for up to 7 days at 4°C for mycotoxin analyses.

Sample preparation and extraction and analysis of aflatoxins: Barley malt samples were ground in a laboratory mill to pass through 0.5 mm to 1.0 mm mesh screen and stored at 2-8°C until analysis. The extraction of aflatoxins involves the use of polar protic organic solvents such as either methanol or acetonitrile or acetone mixed in different proportion with small amounts of water [Bertuzzi et al. 2012]. A ground sample, equivalent to 50 g dry matter was blended with 5 g of sodium chloride and 100 ml mixture of methanol and water (4:1 v/v) at high speed for 1 min using a blender following the method of Ma et al. (2013). The extract was transferred to a conical flask and centrifuged at 300 rpm for 30 minutes to remove any particulates. The supernatant was then filtered through Whatman No. 4 filter paper. The filtrate (10 ml) was passed through an Aflatest Immunoaffinity Column at a rate of 1-2 drops/second. Distilled water (10 ml) was passed through the column at 1-2 drops/second and repeated once more until no bubbles came through the column. Aflatoxin was eluted from the column with 1 ml High Performance Liquid Chromatography (HPLC) grade methanol at the rate of 1-2 drops/second into a glass vial. Two ml of the filtrate was diluted with 14 ml phosphate buffer saline (PBS) passed through an AflaCLEAN Immunoaffinity column at a flow rate of 2 ml per minute. The column was washed with 20 ml of PBS and residual liquid removed by passing clean (oil-free) air through the column for 5 minutes. Aflatoxin was eluted from the immunoaffinity column at a flow rate of 1 drop/second using 1 ml of 100% methanol and de-ionized water respectively and collected in an amber glass vial. The extract was filtered through Millipore 0.45µm prior to injecting in to the HPLC system.

Aflatoxin standards, its identification and quantification:

HPLC grade acetonitrile, methanol, n-hexane, NaCl and deionized water were used for Aflatoxin analysis. AFG₁, AFG₂, AFB₁ and AFB₂ reference standards were obtained from Sigma-Aldrich and stored in dark place at 4°C. Five-point calibration curve graphs were obtained using concentration of 1-16 ppb of AFG₂, 5-80 ppb of AFG₁, 1-16 ppb of AFB₂ and 10-160 ppb of AFB₁. HPLC was used to confirm identity of the Aflatoxin and quantified using Bragulat et al. method (2001) which consists of Shimadzu HPLC system connected to a programmable fluorescence detector. Twenty microliters µl of each extract was applied to HPLC and chromatographic separation was performed on a stainless steel C₁₈ reversed phase Shim-pack FC-ODS column (150 × 4.6 mm, 5 µm particle size) preceded by a C₁₈ pre-column. Water: methanol: acetonitrile (65:25:15, v/v/v) was used as the mobile phase at a flow rate of 1.5 mL/min and 30°C column temperature. Fluorescence of aflatoxin derivatives was recorded at excitation and emission wavelengths of 360 nm and 440 nm respectively. Aflatoxins were quantified based on HPLC fluorometric response compared with aflatoxin standards.

HPLC determination and calculations: The eluate methanol solution aflatoxins (B₁, B₂, G₁ and G₂) were determined at parts per billion (µg/kg) levels by high performance liquid chromatography, immunoaffinity column cleanup and fluorescence detection calculated using the equation:

$$\text{Aflatoxin, } \mu\text{g/kg} = A (T/I) (1/W)$$

Where, A= µg/kg of aflatoxin as eluate injected

T= final test solution eluate volume (ml)

I= volume eluate injected into LC (ml)

W= Commodity mass represented by final extract (g)

Results

Levels of aflatoxins in traditional barley malt samples:

In the present study, samples of traditionally and industrially produced barley malts were collected from three towns in Gojam and three breweries. Samples were analyzed in triplicate duplicate for AFG₂, AFG₁, AFB₂ and AFB₁ contamination by HPLC-FLD. The mean aflatoxin contamination in barley malt samples from Debremarkos were 5.30 µg/kg and the range was 0.0-23.6 µg/kg (Table 1). This was the second highest level of aflatoxin contamination compared with the two study sites. Of all the four aflatoxin types commonly found in food commodities, only aflatoxin G₁ was found to be contaminating barley malt from Debremarkos. The total aflatoxin content of barley malt from Enjibara was the highest (6.5 µg/kg) and the lowest from Finoteselam (1.86µg/kg). Overall, the four types of aflatoxins identified (B₁, B₂, G₁ and G₂) were at higher levels in barley malt from Enjibara than those from Finoteselam. The most common aflatoxin types found at the highest concentration in samples from Enjibara were aflatoxin B₂ at 0.0 - 20.03 µg/kg with a median value of 3.20 µg/kg, aflatoxin G₁ at 0.0 -8.03 µg/kg (median = 1.64) and aflatoxin B₁ at 0.0-5.33 µg/kg (median=1.40 µg/kg). Although barley samples from Finoteselam were contaminated by all four aflatoxin types, their concentrations are much lower.

The percentage of barley malt samples contaminated by aflatoxins is 37.5% both in Debremarkos and Finoteselam and 50% in Enjibara (Table 2). The mean moisture content of the barley malt samples were 6.6, 9.7 and 7.4% for Debremarkos, Finoteselam and Enjibara, respectively.

Table 1: Levels of the different types of aflatoxin identified in malt samples from the three study towns

Types of aflatoxin N=8	Aflatoxin levels (µg/kg)					
	Debremarkos		Enjibara		Finoteselam	
	Range	Median	Range	Median	Range	Median
AFB ₁	ND	ND	0.0-5.33	1.40	0.0-2.95	0.50
AFB ₂	ND	ND	0.0-20.03	3.20	0.0-2.49	0.64
AFG ₁	ND	ND	0.0-8.03	1.64	0-2.60	0.60
AFG ₂	0.0-23.60	5.32	0.0-1.91	0.28	0.0-0.67	0.08
Total median aflatoxin	-	5.32	-	6.50	-	1.86

ND-not detected

Table 2: Percentage of barley malts contaminated with the four types aflatoxins

Sampling site	Total aflatoxin (µg/kg)	Contamination (%)	Mean moisture content (%)
Debremarkos	15.13	37.5	6.6
Enjibara	53.77	50.0	9.7
Finoteselam	42.41	37.5	7.4

Levels of aflatoxin in industrially produced barley malts:

Aflatoxins were not detected in samples collected from brewery 1 and 2. However, brewery 3 has a total aflatoxin content of 3.67µg/kg. Aflatoxin G₁ was detected at 1.97 µg/kg followed by aflatoxin

B₁ at 1.21 µg/kg whereas aflatoxins B₂ and G₂ were detected at much lower concentrations (Table 3). The contamination in imported barley malts collected from breweries 4, 5 and 6 is shown in Table 4.

Table 3: Aflatoxin level of locally produced industrial barley malt ($\mu\text{g/kg}$)

Types of aflatoxin	Sampling sites		
	Brewery 1	Brewery 2	Brewery 3
AFB1	ND	ND	1.21
AFB2	ND	ND	0.21
AFG1	ND	ND	1.97
AFG2	ND	ND	0.28
Total aflatoxin	-	-	3.67

ND-not detected

Table 4: Aflatoxin levels of imported barley malt ($\mu\text{g/kg}$)

Types of aflatoxin	Sampling sites		
	Brewery 4	Brewery 5	Brewery 6
AFB1	ND	ND	1.61
AFB2	ND	ND	0.35
AFG1	ND	ND	2.67
AFG2	ND	ND	0.43
Total aflatoxin	-	-	5.06

ND-not detected

Aflatoxin was detected in brewery 6 only at a total of $5.06 \mu\text{g/kg}$. AFG1 was detected at $2.67 \mu\text{g/kg}$ followed by aflatoxin B1 at $1.61 \mu\text{g/kg}$ whereas

aflatoxins B2 and G2 were detected at much lower concentrations.

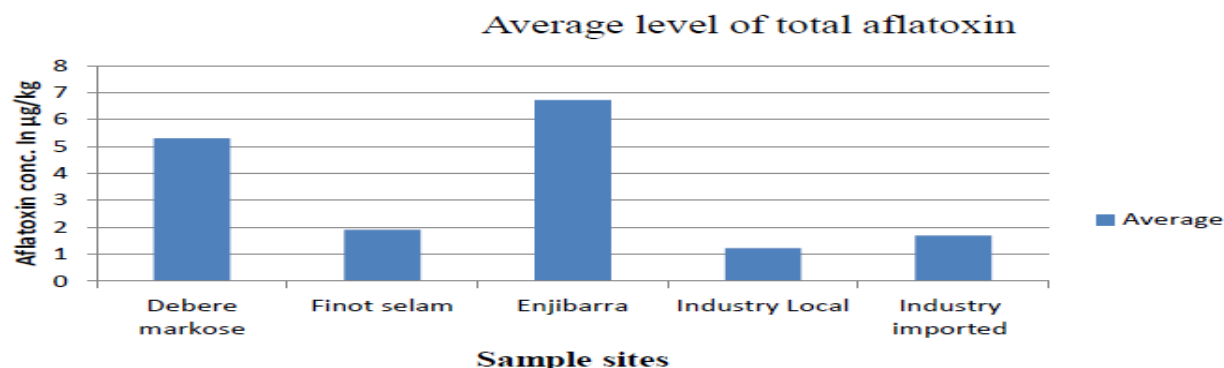


Figure 1: A bar graph for comparing the level of aflatoxin between traditional and industrial barley malt

As depicted in Figure 1, malt barley samples collected from Enjibara town were highly contaminated by aflatoxin followed by samples from Debre markos town. Locally produced industrial barley malt has lower concentration of aflatoxins as compared to imported barley malt.

Discussion

One feature in African cultures is high consumption of home brewed alcoholic beverages. The commonly used ingredients in traditional beer brewing include malted barley, maize, sorghum and finger millet as sources of mylases. However, these cereal grains are prone to pre-harvest and post-harvest contamination with aflatoxins. Mycotoxin production may occur with the growth of mycotoxin-producing fungi during steeping, germination and possibly drying in the malting process (Schwarz et al. 1995b). This is the first study carried out on the levels of aflatoxins in barely malts in Ethiopia. In the present study aflatoxin was detected in malt samples from Debre markos

(37.5%), Finoteselam (37.5%) and Enjibara (50.00%). This could have been attributed by the handling condition of the malts. In the market places of the study towns, where malt is sold, malts are spread on tarpaulins on the ground without cover and this could expose the product to wet and dry conditions. Previously, it was documented that significant correlations existed between weather conditions and aflatoxin levels, whereby a wet and humid climate tends to favor the growth of *Aspergillus* species and increase aflatoxin production in commodities [Hell et al. 2000]. The higher aflatoxin levels in malts from Enjibara town could have been attributed by high moisture contents in malts due to wet and humid climate in the area which increases colonization of *Aspergillus* species and therefore high aflatoxin production compared to other towns with relatively dry weather conditions. The study is in line with a previous study on maize samples (Kaaya and Kyamuhangire 2006).

The four types of aflatoxins identified (B1, B2 and G2) in this study were at higher levels in malts from Finoteselam and Enjibara towns than those from Debreworkos town.

There were high incidences of aflatoxin B1 and B2 types across the samples in both towns, and a significantly higher incidence of aflatoxin type G1 in Debreworkos. Production of aflatoxin G1 type only in malt barley samples from Debreworkos indicates heavy infestation of the samples with *Aspergillus parasiticus* as compared with samples from the other two towns. The result is in line with previous report on peanuts, which documented *Aspergillus parasiticus* as one of the key fungus that contributes to high production of aflatoxin G1 (Hendriks and Jones 2009). In addition to producing Aflatoxin G1 and G2, *Aspergillus parasiticus* is also capable of producing of aflatoxin B1 and B2. This could have contributed to the high proportions of the two aflatoxins types in malt samples particularly those from Finoteselam and Enjibara.

Aflatoxins are thermostable and can pass from contaminated cereals to bakery and cereal (including beer) products including. Therefore, their occurrence is monitored, some of them have been regulated by European legislation. The European Commission has established maximum permitted levels for mycotoxins of major concern in unprocessed cereals other than maize, with 2 µg/kg being the maximum permitted level for AFB1 and 4 µg/kg for the sum of AFB1, AFG1, AFB2 and AFG2 (European Commission 2006).

In the present study, total aflatoxin content of traditional malts from Debreworkos and Enjibara exceed the regulation of the European Commission. When locally produced and imported industrial malts are compared, the aflatoxin content of imported malts exceeds those of industrially produced local malts. The aflatoxin content of industrially produced local malts are below the permitted limits of EU regulation in contrast to imported malts.

Although no studies have been carried out in the aflatoxin content of home-made beer and conventional beer in Ethiopia, home-made beverages particularly those contain relatively low amounts of ethanol may be risk for aflatoxin, because these are sometimes consumed in large quantities (up to 5-6 L/day). In addition, there are possibilities where aflatoxin could be transferred to beer during the brewing process similar to reports found in some types of traditional African beer and from other countries (Odhav and Naicker 2002). These mycotoxins are transferred to the beer during the brewing stages with beer drinkers exposed to these

mycotoxins (Inoue et al. 2013). Sources of mycotoxins in beer could include contaminated malt or adjunct grains used in malting and brewing (Charlene and Wolf-Hall 2007).

Conclusions

The present study provides information on the occurrence of aflatoxins in traditional and industrial barley malts in Ethiopia and the risk of their consumption that poses to aflatoxin exposure.

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