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Research Article

Study on *Aspergillus* Species and Aflatoxin Levels in *Sorghum* (*Sorghum bicolor L.*) Stored for Different Period and Storage System in Kewet Districts, Northern Shewa, Ethiopia

Geremew Tassew Weledesemayat[1], Tilahun Bekele Gezmu[2], Ashagrie Zewdu Woldegiorgis[2] and Habtamu Fekadu Gemede[2,3]*

[1]*Food, Medicine and Health Care Administration and Control Authority of Ethiopia, Addis Ababa, Ethiopia*

[2]*Center for Food Science & Nutrition, College of Natural Sciences, Addis Ababa University, Ethiopia*

[3]*Department of Food Technology and Process Engineering, Wollega University, Nekemte, Ethiopia*

Abstract

Sorghum serves as staple food for over 100 million people in Sub-Saharan African countries. It is the most important nutritional security crop and ranks third among major cereal crops in terms of area and production next to teff and maize in Ethiopia. However, *Sorghum* is susceptible to contamination by molds that produces aflatoxin that causes hepatotoxic and carcinogenic effects on humans and animals. This study was conducted to assess Aspergillus species and aflatoxin level in *Sorghum* (*Sorghum bicolor L.*) stored under different storage system for different storage period. Thirty samples were analyzed for aflatoxin contamination using high performance liquid chromatography equipped with fluorescent detector and *Aspergillus* species were isolated and identified using phenotypic features in a potato dextrose agar culture media. About 56.7%, 16.7%, and 23.3% of the *Sorghum* samples were found to be contaminated with *Aspergillus flavus, Aspergillus niger* and *Aspergillus parasiticus*, respectively. The level of total aflatoxin, aflatoxin B1, aflatoxin B2, aflatoxin G1, and aflatoxin G2 were in the range of 11.44 to 344.26μg/kg, 3.95 to 153.72μg/kg, 1.17 to 91.82μg/kg, 9.87 to 139.64μg/kg, and 3.22 to 52.02μg/kg, respectively. The concentration of aflatoxin in all *Sorghum* samples surpassed the maximum level set by the European commission and therefore, deserves attention to control them across the *Sorghum* value-chain.

Keywords: Aflatoxin; *Aspergillus* species; *Sorghum*; Storage Period; Storage System

***Corresponding author:** Habtamu Fekadu Gemede, Department of Food Technology and Process Engineering, Wollega University, P.O. Box: 395, Nekemte, Ethiopia, Tel: +251 917036924; E-mail: fekadu_habtamu@yahoo.com

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Introduction

Sorghum (*Sorghum bicolor* L. Moench) is the world's fifth most important cereal crop that is grown for grain and fodder in the semi-arid tropics, mainly in Asian and African countries [1]. *Sorghum* is used as a major food and nutritional security crop for more than 100 million people in the Horn of Africa [2]. Ethiopia is one of the major centers of origin and diversity for *Sorghum* cultivation [3]. *Sorghum* ranks third among major cereal crops in terms of area and production next to teff (*Eragrostis* teff) and maize (*Zea mays*) throughout the country [4]. It is estimated more than 1.6 million hectares of the land covered with *Sorghum* production [5]. The lives of millions of Ethiopians depend on *Sorghum* as a staple food crop [1]. However, *Sorghum* crops have been affected by numerous mould contaminations [6].

Aflatoxins are naturally occurring toxic secondary metabolites of the storage fungi (*Aspergillus flavus* and *Aspergillus parasiticus*) which are produced in most agricultural products stored at inappropriate places, temperatures and moisture level. It is extremely persistent under most conditions of storage, handling and processing [7]. These two species are common and widely distributed in tropical and sub-tropical parts of the world. Aflatoxin has been found as contaminant in agricultural food products especially in cereals and animal feeds. *Aspergillus flavus* is common and widespread in nature and is most often found when certain grains are grown under stressful conditions such as drought. Additionally, mould occurs in soil, decaying vegetation, hay and grains undergoing microbiological deterioration and invades all types of organic substrates whenever and wherever the conditions are favorable for its growth [8,9].

Aflatoxin contamination of food causes hepatotoxicity, carcinogenicity, immunosuppression, mutagenic and teratogenic [10] and associated with childhood stunting, which cause severe economical losses to the country [11]. It is a serious health problem because factors which encourage the production of these toxins by mycotoxin (*Aspergillus flavus* and *Aspergillus parasiticus*) abound in Africa. Their presence in food interferes with micronutrients absorption and status in the body and as a consequence, they affect immunity and development [10]. Aflatoxin B1 is one of the most potent naturally occurring animals carcinogen; and immunosuppressive to immunosuppressant [12]. The extent to which mycotoxins affect human health is difficult to investigate in countries where health system lack capacity and resources are limited. For instance, factors such as immune suppression contributing to the overall burden of infectious diseases is difficult to quantify, but is undoubtedly significant. However, food safety remains an important opportunity for addressing current health problems in developing countries [13].

Previous reports have indicated aflatoxin contamination of cereals and pulses such as *Sorghum*, teff, wheat, maize, peanut,

legumes collected from silos, warehouses, shops and market places in Ethiopia [14]. Many investigators have detected aflatoxin and other mycotoxins in many agricultural foods such as maize, teff, broad bean, *Sorghum*, beriberi, traditional spices (mitten shiro) and wheat in Ethiopia [15-20].

Kewet woreda is one of the *Sorghum* producing districts in the country and farmers use both underground pit and above ground storage systems. The storage system "Gotera" are made from different plants, clay, grass, ash, and cow dung. In the underground pit storage, they wash the pit with water and put the *Sorghum* grain until they are full, then the cover with grass, clay and flatted stone. Some farmers are using cleaning, insecticides and fumigants to prevent insect damage and adding the *Sorghum* grain in to the pits. The grain is stored for long periods; especially, this is the case during times of food scarcity. These storage systems are believed to protect against insect damage and theft, fire, domestic and wild animals and improve the quality of *Sorghum* as well. These *Sorghum* grains are stored under unhygienic conditions and very often spoiled by moulds and may develop mycotoxin contamination. Therefore, this particular research was endeavored to analyze the occurrence of *Aspergillus* species and aflatoxin content in *Sorghum* grain stored at different time periods and different storage system.

Materials and Methods

Description of the study area

The sample was collected from North Showa Zone of Amhara region which is located at about 225 km to the north of Addis Ababa along the main road to Dessie. North Showa Zone consists of 26 districts and among these districts; Ataye, Kewet, Merahbete, Meda Oromo and Ensaro are high in *Sorghum* production area. Based on the storage system and periods and production rate, samples were collected from Kewet woreda of 5 Kebeles (Rassa, Ashgne yegeda, Gerenefara, and Teri and Yelen. From each of Kebeles, six role model farmers were proposed and selected on their production rate and one kilogram *Sorghum* sample was collected from each role model farmer. Kewet district is a lowland and semi-arid area that lies at an altitude ranging between 1280 and 2700 meters above sea level. It is located at longitude and latitude of 10˚00'N 39˚54'E and 10.000˚N 39.900˚E, respectively. The vast majority of people are rural small holding who depend on cultivation of 16,046 hectares of arable land. Kewet district has a population size of 100,760. The average annual rainfall is around 600-700mm and temperature of the area ranges from 17 to 30˚C.

Chemicals and reagents

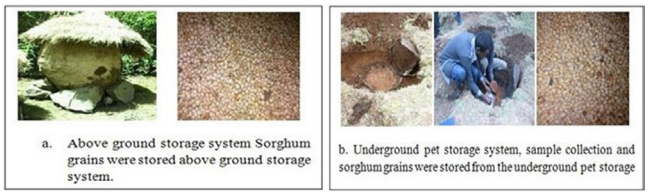
The chemical and reagents used for aflatoxin analysis were HPLC grade. acetonitrile, methanol, n-hexane, $MgSO_4$ anhydrous salt, NaCl, aflatoxin standard and deionized water. The AFG1, AFG2, AFB1 and AFB2 standards were obtained from Sigma-Aldrich. The pure reference standards were stored in dark place at 4˚C. The *Aspergillus* species isolation and identification equipments and chemicals utilized were incubator, petridish and Potato Dextrose Agar (PDA), 10% sodium hypochlorite solution, and ethanol absolute (99.7%).

Survey questionnaire

The survey questionnaire was targeted on educational level, mould contamination, mould color, drying method, storage system, storage time, location of storage, cleaning and sanitation, critical problems and measure taken to control the problems, if any. The role model farmers were chosen purposively based on the information given by the DA (Agricultural Developmental Agents) workers. The farmers were interviewed by means of one to one correspondence.

Sampling and sample size

In this study, a total of 30 samples of *Sorghum* (15 samples from each storage type or 10 samples per each storage period) were collected purposively by taking the storage periods (< 12 month, 1-2 year and ≥ year) and storage system (above ground and underground pit storage systems) into consideration. About 1kg of *Sorghum* samples were collected from the aboveground storage and underground pit storage system from each role model farmers. The samples were collected in plastic bags by taking preventive measures to avoid adventitious contamination and were transported to the laboratory of food science and nutrition at Addis Ababa University. The *Sorghum* grains were separated from foreign matter and milled using miller and sieved to pass through 1mm mesh size. The flour was packaged in tight polyethylene bags and stored in cool dry place. However, whole grains of *Sorghum* were utilized for *Aspergillus* species isolation and identification. The sample collection and sampling were identified figure 1 below.



a. Above ground storage system Sorghum grains were stored above ground storage system.

b. Underground pet storage system, sample collection and sorghum grains were stored from the underground pet storage

Figure 1: About 1kg of *Sorghum* samples were collected from the aboveground storage and underground pit storage system from each selected role model farmer.

Determination of moisture content

Moisture content was determined according to [21] using the official method 925.09. A crucible was dried in an oven at 105˚C for 1 hour and placed in desiccators to cool. The weight of the crucible (W1) was determined. 5 gm samples was weighed in the dry crucible (W2) and dried at 105˚C for 3 hours and after cooling to room temperature in desiccators it was again weighed (W3). The moisture content was determined by using Eq. (2).

$$\text{Moisture content in } \% = \frac{W2 - W3}{W2 - W1} * 100 \qquad (2)$$

Determination of aflatoxin

Method adaptation for analysis of aflatoxin

In order to perform the study on aflatoxin contamination level in the 30 *Sorghum* samples, methodology was performed as described by the QuEChERS method (Quick, Easy, Cheap, Effective, Rugged and Safe), by extraction with Milli Q water: methanol/acetonitrile, $MgSO_4$ and NaCl, followed by centrifugation and filtration, and the quantification was carried out by high-performance liquid chromatography with florescence detector, without derivitization.

High performance liquid chromatography equipped with fluorescence detector was used for analyzing the aflatoxin level of the *Sorghum* samples. The column size was 250mm×4.6mm and the Rheodyne injector size was 20µL. Milli Q water and mixture of acetonitrile and methanol (in the ratio of 71.5/28.5) were used as a mobile phase. The Wavelength florescence detector was set at 440nm. The flow rate was limited to 1.0ml/mm. The parameters selected for

method adaptation were linearity, specificity, and accuracy, limit of detection and quantification and precision.

Sample extraction and clean-up

Extraction of aflatoxin from the *Sorghum* samples were performed according to the method that was validated by Ghent University faculty of Pharmaceutical science, Laboratory of Food Analysis, Belgium, Europe [22]. 1.0g of *Sorghum* flour sample was accurately weighed, 5ml of water was added, vortexed briefly and allowed to stand for 30 minutes. 5ml of extraction solvent (100% ACN) was added and mixed by using a vortex mixer and shacked for 30 minute at using shaker. Weigh 2.0 ± 0.05 g of anhydrous salt $MgSO_4$ and 0.5 ± 0.01g of NaCl and added to the test tube. Shacked immediately to the tubes briefly to prevent agglomeration of the salts and vortex for 2 min, centrifuge at 4000g for 15 min. then transferred 4ml of the top organic layer to a new tube and evaporate under Rotta Vapor and 200µL of injection solvent (A: B 50/50) and 200µL of n-hexane were added to the residue and vortexed immediately to dissolve the residue. Then, dissolved residue was transfer to a centrifuge filter and centrifuge at 10000g for 10 min.

The final extracts were filtered through a 0.45µm PTFE membrane, which is 150µl of the lower phase added in to vials and 20µL were injected into a high performance liquid chromatography column, using a Shimadzu high pressure liquid chromatography (Kyoto, Japan) with fluorescence detector (excitation at 365nm and emission above 440nm).

Isolation and identification of fungi

Fifteen *Sorghum* seeds per sample were surface sterilized with 10% sodium hypochlorite solution for 1 min, followed by immersion in sterile distilled water for 1 min. Surface sterilized seeds were then placed on freshly prepared Potato Dextrose Agar (PDA) plates (ten seeds per plate) and incubated for three days at 25˚C. Pure cultures of different out growing fungi were obtained by transferring fungal colonies to new PDA plates using sterile toothpicks, and incubating the plates for 5-7 days at 25˚C. Pure cultures of each isolate were then stored at 4˚C in test tube containing 2.5ml of sterile distilled water for further use.

Isolates were identified to a species level based on morphological (phenotypic) features as described by [23]. For this purpose: isolates representing each pure culture were grown on PDA Agar at 25˚C for 5-7 days. Fungal colonies that grow rapidly and produce colors of white, yellow, yellow-brown, brown to black or shades of green, mostly consisting of a dense felt of erect conidiophores were broadly classified as *Aspergillus* species. while those that produce blue spores were considered as *Pencillium* species. Isolates with dark green colonies and rough conidia were considered as *Aspergillus parasiticus*. The major distinction currently separating *Aspergillus niger* from the other species of *Aspergillus* is the production of carbon black or very dark brown spores from *Biseriate phialides* [24].

Data analysis

Sorghum samples were analyzed in duplicates and the data obtained were analyzed statistically to calculate the level of significance of various parameters using Analysis of Variance (one way-ANOVA) for storage period and paired comparison for storage system, using the SPSS software version 20.0. The results were reported as mean ± SD and percentages. Least Significant Difference (LSD) was utilized for mean separation and p-value < 0.05 was considered to be significant.

Results and Discussion

Information on farmer's awareness of mould contamination

A total of 30 farmers (4 female and 26 male) were interviewed for their knowledge regarding aflatoxin contamination in their locality. About 14 farmers (46.7%) respondents reported they were aware of mould contamination and the remaining 16 farmers (53.33%) were not. On the other hand, the 26.6% of respondents answered that the color of mould is Black and 6 farmers (20%) responded it as white. The major critical problems in *Sorghum* production and storage identified by the farmers included insects (33.33%), rodent (33.33%) and mould contamination (16.7%). About 75% of the farmers reported they used insecticides to control insect damage to *Sorghum* grains and about

Characteristics	Response	Frequency	Percent (%)
Age	19-38	14	46.7
	38-55	13	33.33
	> 55	3	10
Education	Illiterate	19	63.33
	Primary school	9	30
	Secondary school	2	6.7
Sex	Female	4	13.3
	Male	26	86.7
Mould	Yes	14	46.7
	No	16	53.3
Color	Black	8	26.6
	White	6	20
Problem	Yes	0	0
	No	30	100
Critical problem	Insect	15	50
	Rodent	10	33.33
	Mould	5	16.7
	Other	0	0
Measure taken to control	Insect sides	25	75
	Fire wood smoke	5	25
Drying technology	Sun drying	30	100
	Other technology	0	0
Cleaning and sanitation	Yes	30	100
	No	0	0
Storage Location	Field	15	50
	Inside house	5	16.7
	Courtyard	10	33.3
Mixing	Yes	0	0
	No	30	100
Storage system	Above ground (Gotera)	9	30
	Underground (pit)	10	33.3
	Both (Pit and Gotera)	11	36.7
Duration of storage time periods	Six month	11	36.7
	One year	15	50
	Two year and above	4	13.3
	More than four year	All	100

Table 1: Socio demographic data and farmers' awareness about mould contamination.

5(16.7%) of the farmers described they use other control mechanisms such as fire wood smoking. All farmers in the study area used natural drying method (sun drying) to adjust moisture of the grains prior to storage. With respect to location of the *Sorghum* storage system, 50% of the farmers stored their grains in the field, 16.7% stored inside house and 33.3% stored out in the courtyard. Nine farmers (30%) used above ground storage system and about 10 farmers (33.3%) used underground pit storage and 11 farmers (36.7%) used both aboveground (Gotera) and pit storage system. According to the informants, *Sorghum* can be stored for up to four years (Table 1).

Aflatoxin level in *Sorghum* stored at different storage periods and storage system

All 30 *Sorghum* samples were collected and assayed in duplicate for total aflatoxin contents and the average value for each sample was calculated. The total moisture contents of *Sorghum* samples in the present study were in the range of 8.8-11.86% for *Sorghum* grain stored for different storage periods (< 12 month, 1-2 year and ≥ 2 year) and storage system (aboveground and underground pit storage). An indication of the moisture content and aflatoxin detected in *Sorghum* were shown in table 2.

Aflatoxin B1 and total aflatoxin content in *Sorghum* stored for less than 12 month

The moisture content of *Sorghum* samples stored for less than 12 months in the study was 9.53- 11.4% and 8.77-11.5% for aboveground and underground pit storage, respectively. Aflatoxin contamination levels detected in *Sorghum* samples stored above ground (Gotera) storage system for less than 12 months were in the range of 2.15-27.12µg/kg for AFB2, 3.1-41.96µg/kg for AFG2 and 16.62-139.64µg/kg for AFG1. Sorghum samples stored underground were detected in the range of 14.17-20.38µg/kg for AFB2, 7.46-26.16µg/kg for AFG2 and 16.98-56.39µg/kg for AFG1 (Table 2). The mean aflatoxin B1 content of *Sorghum* stored for less than 12 months above ground (Gotera) was in the range of 3.96-69.79µg/kg which was relatively lower than *Sorghum* stored underground (5.3-82.34µg/kg). The mean total aflatoxin content of *Sorghum* stored for < 12 month above ground was in the range of 16.59-183.16µg/kg and was slightly greater than *Sorghum* stored underground (46.48-177.33µg/kg). Therefore, the study showed that both *Sorghum* grains stored underground pit and aboveground (Gotera) were highly contaminated with aflatoxin. The reason for high aflatoxin content in samples stored aboveground could be because most farmer in the study are use improper construction materials such as clay, grass, caw dung, stone, plane water, ash and *Sorghum* stalk which encourages mould growth. Moreover, farmers repeatedly use the same storage system without properly cleaning it and without drying the *Sorghum* grains.

Aflatoxin B1 and total aflatoxin content in *Sorghum* stored for between one and two year

The moisture content of *Sorghum* samples stored for between one and two year in the current study was 9.33-11.86% and 9.4-12.5% for aboveground (Gotera) and underground pit storage, respectively. Aflatoxin contamination levels detected *Sorghum* samples stored above ground (Gotera) storage system between one and two years were in the range of 2.76-52.05µg/kg for AFB2, 4.65-51.01µg/kg for AFG2 and 18.96-101.79µg/kg for AFG1. Sorghum samples stored underground pit were detected in the range of 3.06-35.01µg/kg for AFB2, 2.04-52.84µg/kg for AFG2 and 42.30-116.13µg/kg for AFG1 (Table 2). The mean aflatoxin B1 content of *Sorghum* stored between one and two year above ground storage (Gotera) was in the range of 21.75-88.90µg/kg which was relatively lower than *Sorghum* stored for underground (3.95-153.72µg/kg). The mean total aflatoxin content of *Sorghum* stored for between one and two year above ground storage was in the range of 44.27-210.53µg/kg and was relatively lower than *Sorghum* stored underground (11.44-344.26µg/kg). Therefore, the study showed that the *Sorghum* grain stored underground was highly infected by mould and hence contained higher aflatoxin concentration. The reason for high aflatoxin contamination in *Sorghum* stored underground could be because most farmers in the study area had misunderstanding about mould color and *Sorghum* handling.

Aflatoxin B1 and total aflatoxin content in *Sorghum* stored for two or more than year

The moisture content of *Sorghum* samples stored for two or more than years in the present study were 8.9- 11.3% and 9.4-11.75% for aboveground and underground pit storage, respectively. Aflatoxin contamination levels detected *Sorghum* samples stored aboveground for two or more years were in the range of 1.17-91.82µg/kg for AFB2, 16.95-72.65µg/kg for AFG2 and 12.92-114.10µg/kg for AFG1. Sorghum samples stored in underground pit were detected in the range of 6.89-21.35µg/kg for AFB2, 3.22-12.58µg/kg for AFG2 and 9.87-129.26µg/kg for AFG1 (Table 2). The mean aflatoxin B1 content of *Sorghum* stored above ground for two or more years was in the range of 12.95-105.96µg/kg which was relatively higher than *Sorghum* stored underground pit storage (4.59-15.57µg/kg). The mean total aflatoxin content of *Sorghum* stored for two or more years aboveground (Gotera) was in the range of 65.17-247.92µg/kg and was relatively higher than *Sorghum* stored underground (17.68-164.3µg/kg). Therefore, the study showed that *Sorghum* grain stored aboveground (Gotera) storage was highly susceptible to aflatoxin contamination. The reasons for high aflatoxin contamination levels in the *Sorghum* sample stored aboveground (Gotera) could be because of most farmers in the study area use poor cultivation and harvesting, inadequate handling for example drying, insect control, cleaning, and storage practice.

From the total 30 samples, 96.66%, 93.33%, 96.7%, and 90% were contaminated by aflatoxin B1, B2, G2 and G1, respectively. These showed total aflatoxin contamination by the level ranged from 11.44µg/kg to 344.26µg/kg and the mean total aflatoxin value of 123.85µg/kg. Aflatoxin contamination levels were detected *Sorghum* samples in range of 1.17-91.82µg/kg for AFB2, 3.22-139.64µg/kg for AFG2 and 9.87-139.64µg/kg for AFG1 (Table 2). Enormous variation of aflatoxin contamination was observed between 23 *Sorghum* samples and 7 samples had aflatoxin levels below detection limits (3 for AFG1, 2 for AFB2, 1 for AFB1 and AFG2). This may be due to the variation in fungal colonization, especially *Aspergillus flavus*, and spore density during the grain development stage. It had comparable to the variation of this fungus in developing *Sorghum* grain was studied earlier by Ratnavathi et al. [25].

About 96.66% of the total aflatoxin in the *Sorghum* samples was attributed to AFB1 which ranged from 3.95- 153.72µg/kg. Generally speaking, AFB1 is the most toxic aflatoxin among all types of aflatoxin and is considered to be hepatocarcinogenic and immunosuppressive as well [12]. Therefore, the high concentration of the AFB1 in *Sorghum* samples indicates that there could be a serious problem of aflatoxin contaminations in the study area.

Storage system	Sample Code	B1 (µg/kg)	B2 (µg/kg)	G2 (µg/kg)	G1 (µg/kg)	Total Afs (µg/kg)	Moisture content (%)
< 12 month stored *Sorghum*							
Aboveground storage	S01	28.82	11.17	3.1	16.62	75.57	11.4
	S02	3.96	4.29	50.2	111.25	16.59	10.4
	S03	31.76	2.15	7.65	24.94	60.41	9.73
	S04	71.52	22.81	26.25	139.64	176.49	9.53
	S05	69.79	27.12	41.96	113.36	183.16	10.2
Underground pit storage	S06	82.34	14.77	8.44	16.98	177.33	11.3
	S07	31.49	20.38	7.46	56.39	100.97	10.33
	S08	28.64	18.55	26.16	31.11	91.85	10.43
	S09	28.8	19.11	23.07	50.31	93.4	8.77
	SO10	5.3	16.54	8.47	17.24	46.48	11.53
1-2 year stored *Sorghum*							
Aboveground storage	SO11	21.75	2.76	21.02	101.79	44.27	11.86
	SO12	46.16	3.48	15.73	68.3	88.61	11.43
	SO13	88.9	24.43	51.01	ND	210.53	10.78
	SO14	33.17	52.05	26.28	18.96	175.16	9.82
	SO15	31.06	40.56	4.65	23.87	145.61	9.33
Underground pit storage	SO16	8.86	35.01	12.65	116.13	94.28	11.05
	SO17	3.95	2.01	2.04	80.7	11.44	12.53
	SO18	64.68	17.33	18.74	42.3	152.19	9.47
	SO19	69.69	3.06	52.84	61.82	128.86	10.8
	SO20	153.72	33.45	37.89	ND	344.26	9.4
≥ 2 year stored *Sorghum*							
Aboveground storage	SO21	26.27	6.58	19.4	12.92	65.17	9.4
	SO22	51.28	1.17	32.91	114.1	199.46	9.4
	SO23	12.95	30.26	16.95	78.17	138.33	8.9
	SO24	105.96	91.82	24.61	25.53	247.92	9.19
	SO25	36.75	2.85	72.65	109.2	221.45	11.13
Underground pit storage	SO26	15.57	6.89	12.58	129.26	164.3	9.6
	SO27	9.6	21.35	15.62	51.79	98.36	8.8
	SO28	4.59	10.08	3.22	9.87	17.68	11.75
	SO29	7.25	ND	4.28	9.94	21.47	10.72
	SO30	ND	ND	ND	ND	ND	9.4
% of contamination level		96.66	93.33	96.66	90	-----	-----

Table 2: Level of aflatoxin content in Sorghum sample stored for different period and storage system.

ND -means not detected.

The incidence of aflatoxin contamination in the examined *Sorghum* grain samples appear to be lower than those reported for *Sorghum* and maize [16] in which the aflatoxin contamination reached up to 1000µg/kg for *Sorghum* and 1388µg/kg for wheat, respectively. However, this study result showed a very high aflatoxin levels above the permissible limits. The maximum aflatoxin B1 content found in this research (153.72µg/kg) was below the highest amount reported by Habtam and Kelbesa (692µg/kg) [17]. On other hand, the presence of aflatoxin contamination (96.6%) reported in the present study was relatively higher than the previous study reported by Ayalew [26] aflatoxin infection (88%) in maize from Ethiopia.

The presence of aflatoxin B1 detected in the present study (153.72µg/kg) was generally higher than from the previous study by Chala et al., [15] on *Sorghum* in Ethiopia (29.5µg/kg). However, the amount of aflatoxin B1 (153.72µg/kg) in the *Sorghum* samples in this research was much relatively lower than the finding reported by Alpert et al., [27] which was as high as 1000µg/kg. The concentrations of aflatoxin G1 obtained in the present study were larger (139.64µg/kg) when compared with previous reports for *Sorghum* grain (29.65µg/kg) by Chala et al. [15].

On other hand, the percent of AFB1 level (96.66%) reported in the present study was relatively higher than the previous study reported in Brazil which was 39% for pre-fermented and 32% for post-fermented *Sorghum* samples [28] with maximum values of 5.10µg/kg and 30.05µg/kg, respectively. Therefore, the current study also showed aflatoxin contamination in *Sorghum* stored under different storage system and period. This indicates that there is high problem of aflatoxin contamination in the country.

Effects of storage periods on the level of aflatoxin contamination in *Sorghum*

Sorghum stored for two or more years had high level of aflatoxin B1 (52.19µg/kg) followed by *Sorghum* stored for less than 12 months (38.24µg/kg). Although storage period had no significant (P > 0.05) effect on the level of most of the aflatoxin, it resulted in significant difference in the level of aflatoxin B1 in *Sorghum*. Significant difference (P < 0.05) in the aflatoxin B1 was observed between *Sorghum* grains stored for up to two years (52.19µg/kg) and stored for more than two years (27.02µg/kg). This indicates that storing *Sorghum* for a period between one and two years result in higher contamination by aflatoxin B1. However, *Sorghum* stored for over two years had high level of aflatoxin B2 (21.42µg/kg) and *Sorghum* stored for less than 12 months had high concentration of the aflatoxin G1 (57.7µg/kg). Sorghum grains stored for above two year had high level of total aflatoxin than the remaining storage periods but with no significant difference (P > 0.05) (Table 3). Based on the result of the present study, aflatoxin contamination can occur in grains stored for any storage period given no proper *Sorghum* cultivation, harvesting, transporting, trashing, cleaning, insect prevention and storage practices.

Duration of storage period	Aflatoxin content (µg/kg)				
	AFG2	AFG1	AFB2	AFB1	Total aflatoxin
< 12 months	20.28a	57.78a	15.69a	38.24ab	64.31a
1-2 years	24.28a	51.39a	17.09a	52.19a	59.31a
> 2 years	22.22a	54.08a	21.42a	27.02b	81.64a

Table 3: Effect of storage period (< 12 month, 1-2 year, and ≤ 2 year) on the level of aflatoxin (µg/kg) in *Sorghum*.

Means within the same column followed by the same letter superscript indicate no significant difference (p > 0.05).

The level of aflatoxin B1 contamination (52.19µg/kg) in *Sorghum* storage period (< 12 month, 1-2 year, and ≤ 2 year) were lower than AFB1 in ground pepper, shiro, legumes and spices (incidences of AFB1 contamination occur 13.33% and 8.33%, and the levels of contamination range 250 to 525µg/kg and 100 to 500µg/kg, respectively) previously stated by Habtamu and Kelbessa [29].

However, the aflatoxin content and mycotoxin development in *Sorghum* grain stored for different period may increase due to moisture migration from the surrounding and storage condition (temperature and humidity) reported by Mohamed et al., Mashilla et al., and Habtamu and Kelbessa [17,30,31]. The level of aflatoxin increment stated by Shephard [13], flood damage to grain (mainly *Sorghum*) in underground storage areas resulted in visible fungal contamination and these harsh realities; it is not surprising that fungal contamination of staple food. The current study showed that the study area has a suitable condition for mycotoxin development and farmers may not used sufficiently good handling, harvesting and storage practice. This practice may lead to elevated aflatoxin production.

Effects of storage system on the level of aflatoxin contamination in *Sorghum*

Storage of grains under poor storage system and conditions often result in aflatoxin contaminations. However, in this study, storage system did not result in significant variation between the mean aflatoxin contents of *Sorghum* grains. Grains stored above ground had high aflatoxin level of 49.67µg/kg (G2), 63.91µg/kg (G1), 21.57µg/kg (B2) and 44.0µg/kg (B1) than grains stored in the underground pit which in that order contained 30.05µg/kg, 44.92µg/kg, 14.56µg/kg and 34.30µg/kg. Storage type also did not significantly affect the level of total aflatoxin (p > 0.05), but the level of aflatoxin in storage type still larger than the maximum level stated by EU for aflatoxin B1 (5µg/kg) and for total aflatoxin (10µg/kg) and FDA established maximum acceptable level of 20µg/kg for aflatoxin in maize, and *Sorghum* (Table 4).

The reason for aflatoxin level increment in the current study may be factors, including soil quality, crop yields, and the biological environment of crops such as the abundance of pests and plant pathogens determine the mould growth of stored *Sorghum* [30]. These factors encourage mycotoxin invasion of grains and thereby impart a food-borne risks. Basically, the ability of fungi to produce mycotoxins is largely influenced by temperature, relative humidity, insect attack, and stress conditions of the plants.

Storage type	Average aflatoxin content (µg/kg)			
	AFG2	AFG1	AFB2	AFB1
Above ground	49.67a	63.91a	21.57a	44.1a
Underground pit	30.05a	44.92a	14.56a	34.3a

Table 4: Effect of storage system (above ground and underground pit) on the level of aflatoxin.

Means within the same column followed by the same letter superscript indicate no significant difference (p > 0.05).

HPLC chromatogram of the aflatoxin in *Sorghum* sample

A typical HPLC chromatogram showing the clear separation of 1 ppb standard mixture of four aflatoxins with respect to retention time G2 (19 min), G1 (21 min), B2 (23.5 min) and B1 (25.8 min) and the standard curve, black sample, mould infected sample and *Sorghum* sample was described in figure 2 and figure 3.

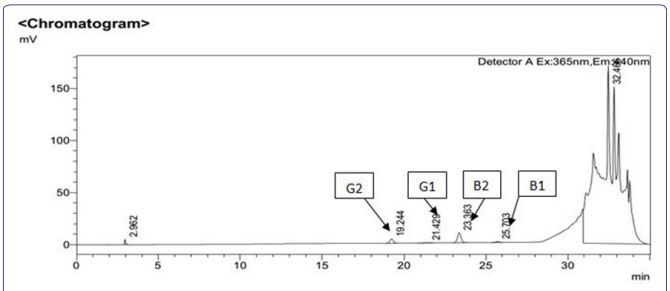


Figure 2: HPLC Chromatogram of 1 ppb standard mixture of four aflatoxin.

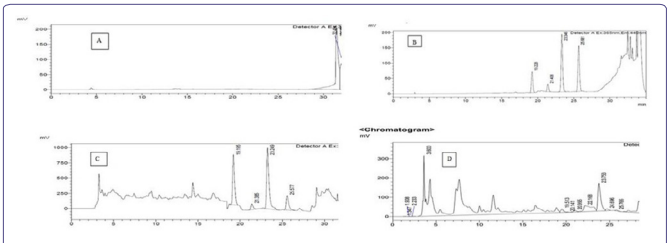


Figure 3: Chromatogram of blank sample (A), standard calibration curve (B) and naturally contaminated fungal sample (peanut) (C), *Sorghum* sample (D), (elution order AFG2, AFG1, AFB2 and AFB1).

Isolation and identification of *Aspergillus* Species from *Sorghum* sample

Aspergillus species isolation representing each pure culture was grown on PDA Agar at 25˚C for 5-7 days. The first species isolated from the *Sorghum* samples was *Aspergillus niger*. The major distinction of separating *Aspergillus niger* from the other species of

Aspergillus is the production of carbon black or dark brown spores of *Biseriate phialides* were indicated as plate 7. *Aspergillus flavus* was the second species identified in this study. Colonies of this fungus were characterized by yellow to dark, yellowish-green pigments, consisting of a dense felt of conidiophores or mature vesicles bearing phialides over their entire surface. In general, A*spergillus flavus* was known as a velvety, yellow to green or the old colony was brown mould with a goldish to red-brown on the reverse. *Aspergillus parasiticus* was the third species identified from *Sorghum* samples tested in the current study. Colonies representing this species produced dark green and rough conidia on PDA at 25 and 37˚C after 5-7 days of incubation. The three identified *Aspergillus species* were indicated in figure 4 below.

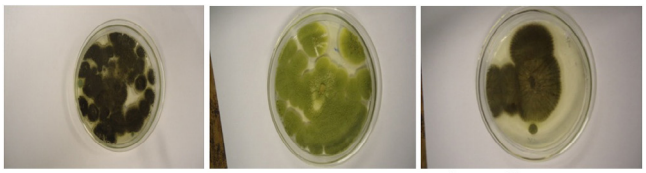


Figure 4: Morphological (phenotypic) feature of *Aspergillus* species, (Photo by-Author).

In this study all *Sorghum* samples come from regions with temperatures ranging from 17 to 30˚C which supports the growth of *Aspergillus* species. Under tropical condition, stored products are more susceptible to *Aspergillus* species than other fungi as many *Aspergillus species* are favored by the combination of low water activity (aw) and relatively high storage temperature [17]. Although, cereal grains belong to corn, rice, barley; wheat and sorghum are found susceptible to aflatoxin accumulation by aflatoxogenic fungus.

In table 5 indicated that the occurrence of *Aspergillus* spp. in *Sorghum* grain stored at different storage period and storage system; 56.7%, 16.7% and 23.33% of *Aspergillus flavus*, *Aspergillus parasiticus* and *Aspergillus niger* was found, respectively from the total 30 samples.

***Aspergillus* species**	**Number of sample**	**% of *Aspergillus* species found**
Aspergillus flavus	17	56.66
Aspergillus parasiticus	5	16.66
Aspergillus niger	7	23.33
Total	29	96.65

Table 5: *Aspergillus* species identified from *Sorghum* sample using PDA agar.

The incidence of *Aspergillus flavus* (56.7%) in the present study was bigger than the occurrences of *Aspergillus flavus* in maize samples from Ethiopia were indicated by Ayalew [26]. However, the invention of *Aspergillus fulvous* (56.7%) in *Sorghum* sample was lower than the occurrence of *Aspergillus flavus* (72.7%) in *Sorghum* grain reported from India [32]. Therefore the occurrence of aflatoxin content in *Sorghum* sample is associated with *Aspergillus flavus* and *Aspergillus parasiticus.* These indicate aflatoxin contaminations in *Sorghum* sample collected from the study area is highly significant.

However, the current results showed that *Sorghum* was more profoundly colonized by aflatoxin producing *Aspergillus* species, with overall aflatoxin levels being correspondingly higher. The *Sorghum* grain contamination by *Aspergillus* species. and the production of aflatoxin are highly influenced by the weather conditions prevailing during the grain development stage, i.e., seed set to physiological maturity stage [25]. In addition, this may be caused by the variations in cultivars, storage periods, and storage system and over all handling practices used.

Table 6 showed that the *Aspergillus flavus* (13.33% for < 12 months 23.33% for 1-2year and 20% for ≥ 2 year) and *Aspergillus parasiticus* (6.67% for < 12 months 6.67% for 1-2 year and 3.33% for ≥ 2 year) were occurred *Sorghum* stored at different periods. *Aspergillus flavus* and *Aspergillus parasiticus* stored at 1-2 year was slightly larger than *Sorghum* grains stored with ≥ 2 year and < 12 month. This indicated that aflatoxin contamination in *Sorghum* grain was highly disposed from storage periods. The presence of *Aspergillus niger* (3.3%) in *Sorghum* stored between one and two year had lower than both 10% of *Aspergillus flavus* and *Aspergillus parasiticus.* Sorghum stored with two or more years had relatively lower *Aspergillus parasiticus* (3.33%) than both 6.67% of *Aspergillus parasiticus* at *Sorghum* stored in less than 12 month and between one and two year.

Duration of storage period	***Aspergillus* species (%)**		
	Aspergillus flavus	***Aspergillus parasiticus***	***Aspergillus niger***
< 12 months	13.33	6.67	10
1-2year	23.33	6.67	3.33
≥ 2 year	20	3.33	10

Table 6: The occurrence of *Aspergillus* species in different storage periods.

Evaluation of aflatoxin results against different international standards

The maximum tolerable limits of aflatoxins in foodstuffs are laid by European Union legislation are specified for aflatoxin B1 (5µg/kg) and maximum total aflatoxin (10µg/kg) [33]. Another regulation, the US Food and Drug Administration (FDA) have established maximum acceptable level of 20 ppb for aflatoxin in maize, *Sorghum* and other cereals for human consumption, with the level in milk being even lower (0.5 ppb) [34]. Likewise, FAO underline the maximum tolerated levels limit of 20µg/kg for mycotoxins in foodstuffs, Maize, kidney beans, rice, *Sorghum*, groundnuts and groundnut butter. Based on the result of the current study, the mean average of aflatoxin concentration in range of AFB1 (3.95-153.72µg/kg) and total aflatoxin (11.44-344.26µg/kg) is higher than the EU regulation limit and FDA maximum tolerable limit.

The aflatoxin content measured in all the *Sorghum* samples showed that natural aflatoxin was higher and 96.66% of samples were contaminated by toxin as compared to highly susceptible crops like maize and groundnut [35]. However, the result also above the safety limit (20µg/kg) recommended by the Codex Alimentarius Committee.

In East African standard specification (CD-ARS 462:2012 (E) for *Sorghum*, it is stated that the *Sorghum* grains shall comply with those maximum mycotoxin limits established by the Codex Alimentarius Commission. In particular, total aflatoxin levels in *Sorghum* grains for human consumption shall not exceed 10µg/kg with AFB1 not exceeding 5µg/kg when tested according to ISO 16050. However, the present study showed that the total aflatoxin concentration and AFB1 were surpassed the specified tolerable levels by the above mentioned regulations.

Conclusion

The level of total aflatoxin in *Sorghum* samples was above tolerable limits set by different organizations. This can be more

hazardous to individuals who are more sensitive and prone to toxic effects of such highly carcinogenic food contaminants. Therefore, this situation clearly demands wider national or international programs for the control of aflatoxin contamination in *Sorghum*. In conclusion, aflatoxin control programs should focus on addressing all the factors that contribute to fungal growth across the value chain (i.e., pre-harvest to household practices). Hence, the concepts like HACCP "Farm to Fork" should be applied.

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