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Occurrence of Aflatoxins in Red pepper (Capsicum *annum L.)*: In Relation to Storage Duration and Quality Grading

BY

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List of Abbreviations and Acronyms

AAU	: Addis Ababa University
AF	: Aflatoxins
AFB1	: Aflatoxin B1
AFB2	: Aflatoxin B2
AFG1	: Aflatoxin G1
AFG2	: Aflatoxin G2
AOAC	: Association of Official Analytical Chemists
CSA	: Central Statistical Authority
ELISA	: Enzyme Linked Immunosorbent Assay
EU	: European Unions
FAO	: Food and Agriculture Organization
HCC	: HepatoCellular Carcinoma
HPLC	: High Performance Liquid Chromatography
IARC	: International Agency for Research on Cancer
JECFA	: Joint Expert Committee on Food Additives
PDA	: Potato Dextros Agar
ppb	: parts per billion
PP bag	: polypropylene bags
RSD	: Relative Standard Deviation
RASFF	: Rappid Alert System for Food and Feed
TLC	: Thin Layer Chromatography
WHO	: World Health Organization

ABSTRACT

Aflatoxin, a contaminant produced by the fungi Aspergillus flavus and Aspergillus parasiticus in several agricultural commodities, is a known human liver carcinogen. Red pepper is widely consumed in Ethiopia and this agricultural commodity is known to be susceptible to aflatoxin contamination. In this research, aflatoxin contamination of whole and ground red pepper in relation with storage duration and quality grading and the magnitude of the contamination in grounded red pepper were studied. Samples were collected from farmers and wholesalers and Aflatoxins were determined using HPLC. Whole red pepper samples stored for three months shows low aflatoxin contamination compared to red pepper samples stored for six months ($p<0.05$).The mean aflatoxin content increases from 19.56 μg/kg to 78.83μg/kg within three months of storage after harvest. Low quality graded red pepper showed significantly high level of contamination ($p<0.05$) compared to high quality graded red pepper. The mean aflatoxin content found was 78.8 μg/kg for Grade1, 160.11 μg/kg for Grade 2 and 312.33 μg/kg for Grade 3. The highest aflatoxin content was found in the least grade level (Grade 3) with a concentration of 399.19μg/kg individual values ranges from 70.80 μg/kg to 399.19 μg/kg. The amount of aflatoxin in grounded red pepper traded in retail stores ranges from 18.37μg/kg to 158.87μg/kg with a mean of 66.34μg/kg. All the grounded samples surpassed the maximum level set by the European Commission (10 μg/kg) for total aflatoxins and (5μg/kg) for aflatoxin B1.The observed practices of sprinkling water on whole red pepper pods by merchants was identified as possible factor for high level of contamination. The result of this study demonstrates that aflatoxin contamination in red pepper is high and could be a health threat. The level of contamination is distinct with respect to quality. As a result, the practice of the merchants should be taken in to account when dealing with aflatoxin management for red pepper in Ethiopia. Moreover, the use of high quality red peppers should be practiced whenever possible as Grade 1 red peppers shows relatively low aflatoxin contamination compared to red pepper traded as Grade 2 and Grade 3.

Key words: *Red pepper, fungi, mycotoxins, aflatoxins, storage duration, quality grade level*

1. Introduction

Aflatoxin(AF), a contaminant mostly produced by the fungi *Aspergillus flavus* and *Aspergillus parasiticus* in several agricultural commodities, is a known human liver carcinogen. AFs have commonly been found to contaminate a wide variety of important food stuffs such as peanuts, corn, rice, wheat, cottonseed and spices. Aflatoxin B_1 is the most acutely toxic and has been classified as group 1 carcinogen, primarily affecting liver by The International Agency for Research on Cancer (IARC, 1993). In the developing world, where climatic conditions and poor storage practices are conducive to fungal growth and then subsequent mycotoxin production, mycotoxin exposure in these populations is high (Shepard, 2008).According to a research, consumption of aflatoxin contaminated foods leads to different liver related diseases including hepatocellular carcinoma (HCC), or liver cancer (Nissen & Martin ,2002).

Moreover, acute illness and death, immune system suppression, stunting and several other nutritional illnesses are also related to dietary exposure to aflatoxins (USAID, 2012).The extent to which mycotoxins affect human health is difficult to investigate in countries whose health systems lack capacity and in which resources are limited. For example, factors such as immune suppression contribute to the overall burden of infectious disease is difficult to quantify, but is undoubtedly significant. Thus, food safety remains an important opportunity for addressing current health problems in developing countries (Shepard, 2008).

Among food stuffs which were studied for mycotoxin occurrence, red pepper is one that is found conducive for fungal growth and is found susceptible for subsequent mycotoxin contamination under conditions prevailing fungal growth (Iqbal et al, 2013). Poor harvesting practices, improper drying, handling, packaging, storage and transport of chillies facilitate fungal growth

and thus results in the increased risk of AFs contamination (Christensen et al, 1967, Flannigan & Hui, 1976, Scott and Keneddy, 1973).

In Ethiopia, red Peppers are widely cultivated in Mareko (SNNPR), Alaba (SNNPR), Ziway (East Shewa), Dembi Dollo (West Wellega), Todalle (Jima Route), Gojam-Gonder. In 2009/2010 report of the Ethiopia Central statistical Agency on production of crops, 1,593,275 Quintals of red pepper was produced for the "Meher "season (September-December) (Central Statistical Agency of Ethiopia, 2010).

Ethiopians have strong attachment to dark red pepper, which has high value principally for its high pungency. The fine powdered pungent product is an indispensable flavoring and coloring ingredient in the common traditional sauce "*Wot*" whereas; the green pod is consumed as a vegetable with other food items. There is a general belief among Ethiopians that a person who frequently consumes hot pepper has resistance to various diseases. As a result, it is common to find red pepper in the daily diet of most Ethiopians (Rehima, 2006).

Preliminary surveys which were made on aflatoxin contamination of ground red pepper showed the presence of the contamination (Fufa and Urga, 1996, Fufa & Urga, 2001, Besrat & Gebre, 1981). Moreover in a study made on primary carcinoma of the liver in Ethiopia, mycotoxins and other hepatotoxic agents suggested as being the most important casual factors (Pavlica & Samuel, 1970).As a result studying a foodstuff which is widely consumed in the country for its mycotoxin level is undoubtedly important.

In addition to the health threat that this commodity could pose due to aflatoxins contamination, the country is facing problems in exporting red pepper as the contamination will prevent the commodity from meeting international and regional regulations and standards governing agricultural trade and food safety (Rehima, 2006).

According to The Rapid Alert System for Food and Feed (RASFF) of the European Union a consignment of chili powder of Ethiopian origin which was exported to the United Kingdom had been detained and destructed before it enters to UK in 2008. The consignment has B1=12μg/kg and Total=32.1 μg/kg (Rapid Alert System for Food and Feed, 2008).

As red pepper is a widely consumed commodity in Ethiopia, it is important to study the contamination level thoroughly in order to reduce the health risk of consumers due to consumption of this commodity. The aim of this research was to determine aflatoxin content of red pepper in relation to quality grading and storage duration in whole and ground red pepper from Mareko which is the major production site in Ethiopia.

2. Objectives

2.1.General objectives

To determine aflatoxin content of red pepper in relation to quality grading and storage duration in whole red pepper and to assess the magnitude of the contamination in ground red pepper traded in retail stores.

2.2.Specific objectives

1. To determine aflatoxin (B_1, G_1, G_2, $G_1$and Total aflatoxin) content in whole red pepper stored for 3 months and 6 months.

2. To see the difference in relation with aflatoxin contamination level among red peppers traded as high quality(Gade-1), low quality (Grad -2) and very low quality(Grade-3)

3. To isolate aflatoxin producing mycroflora and see aflatoxin production potential in red pepper.

4. To assess the level of aflatoxin content in powdered red pepper traded in retail stores.

3. Literature review

3.1.History of red pepper

Origin

Chillies are known from pre-historic times in Peru. They are believed to have originated in the tropical America. It is also said that chilies have originated in the Latin American regions of the New Mexico and Guatemala as a wild crop around 7500BC, as per the remains of the pre-historic Peru. The people native to these places domesticated this crop in and around 5000 BC,. Chili is said to be the first ever domesticated crop in America. The three species *C. annuum*, *C. frutescens* and *C. chinense* evolved from a common ancestor located in the North of the Amazon basin (NW-Brazil, Columbia) (Government of India Ministry of Agriculture, 2009)

Botanical Description

Chilli is a fruit of the plants ***'Capsicum annuum'*** and ***'Capsicum frutecens'*** that come from the genus 'Capsicum,' belonging to the family of 'Solanaceae,' which also includes tomato and potato. Capsicum is derived from the Greek word "Kapsimo" meaning "to bite." Chillies are referred to as chillies, chile, hot peppers, bell peppers, red peppers, pod peppers, cayenne peppers, paprika, pimento, and capsicum in different parts of the world (Government of India Ministry of Agriculture, 2009)

3.2.Nature of aflatoxins and aflatoxigenic fungi

Aflatoxins are produced as secondary metabolites by the fungi *A. flavus* and *A. paraciticus* that contaminate various foods and feeding products (Andrallos et al, 1967). *Aspergillus* is a large genus composed of more than 180 anamorphic species. *Aspergillus* subgenus *Circumdati* section

flavi, also refers as the *Aspergillus flavus* group, and has attracted worldwide attention for its toxigenic potential (Pitt et al, 1998).

Section *Flavi* is divided in two groups of species. One include the aflatoxigenic species *A. flavus, A. parasiticus* and more recently *A. nomius* which cause serious problems worldwide in agricultural commodities, and the other includes the non-aflatoxigenic species *A. oryzae*, *A. sojae* and *A. tamari*, traditionally used for production of fermented foods in Asia (Kumeda & Asao,2001).

Among 18 different types of aflatoxins identified, the major members are aflatoxin B1, B2, G1 and G2. Aflatoxins are fluorescence under the UV light. Aflatoxin B1 and B2 emit blue fluorescence whereas aflatoxin G1 and G2 emit green fluorescence and distinguished on the basis of their blue or green fluorescence as aflatoxins B or G respectively and designated as aflatoxins B1, B2, G1, and G2 in order of decreasing Retention value in paper chromatogram.The quantity and relative proportion of four compounds in culture extracts varies depending on mold strain, medium composition and culture conditions. Normally, aflatoxin B1 is present in largest amounts whereas B2 and G2 are produced in small quantity (Wogan, 1966).

The molecular formula of aflatoxin B1 and G1 were established as $C_{17}H_{12}O_6$ and $C_{17}H_{12}O_7$ respectively. Aflatoxins B2 and G2 are the dihydro derivatives of the parent compounds and molecular formulae of these are $C_{17}H_{14}O_6$ and $C_{17}H_{14}O_6$, respectively. All four aflatoxins have high melting points. The chemical structure of aflatoxin B1, B2, G1 and G2 were proposed in 1963 (Wogan, 1966). These structures are shown in Fig.1.

All compounds are coumarin derivatives that fused to bifuran moiety. Aflatoxin B contains pentanone structure which in placed with six member lactone in aflatoxin G. Some physical properties and spectral characteristics of the compound are summarized in Table 1.

The ultraviolet and infrared absorption spectra are similar in all four compounds. The maximum fluorescence emission of aflatoxin B1 and B2 are 425 nm, and for aflatoxin G1 and G2 are 450 nm. The emission property can be used for estimation of aflatoxins concentration by fluoresce technique (Sobolev, 2007).

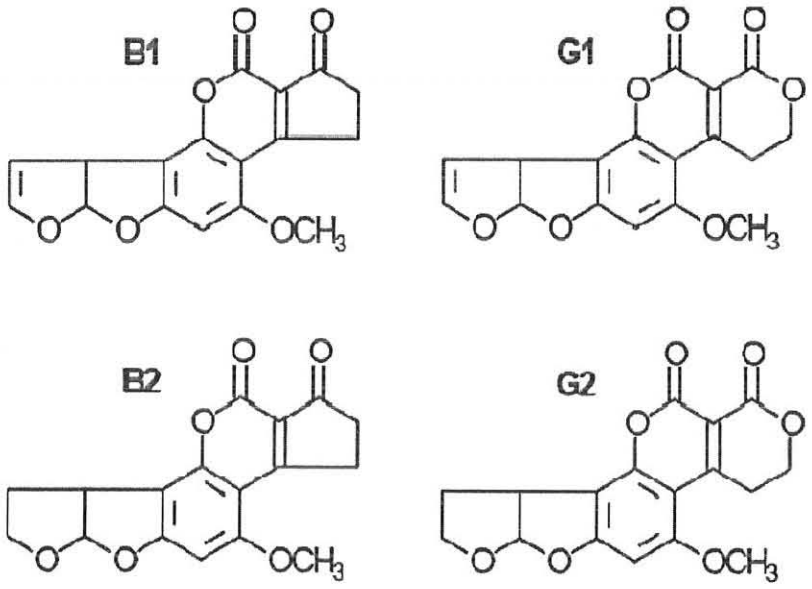

Figure 1.Chemical structures of aflatoxins
Source: - *Wogan, 1966*

Table 1. Summary of physical properties and spectral characterstics of aflatoxins

Aflatoxin	Molecular Formula	Molecular weight	Melting point	Ultraviolet absorption (E)	Infrared absorption (cm-1)	Fluorescence emission
B1	$C_{17}H_{12}O_6$	312	268-269	13,400	21,800	1760/1684/1632/1598
B2	$C_{17}H_{14}O_6$	314	286-289	9,200	14,700	1760/1685/1625/1600
G1	$C_{17}H_{12}O_7$	328	244-246	10,000	16,100	1760/1695/1630/1595
G2	$C_{17}H_{14}O_7$	330	237-240	11,200	19,300	1760/1694/1627/1597

Source*: - Wogan, 1966*

3.3.Health effects and toxicity of aflatoxins

Acute aflatoxin poisoning is caused by ingestion of high levels of the toxin. Immediate consequences are severe liver damage, acute jaundice and hepatitis, which subsequently may result in death (Bennett and Klich, 2003).The principal target organ for aflatoxicosis is the liver. After the invasion of aflatoxins into the liver, lipids infiltrate hepatocytes and leads to necrosis or liver cell death. Other general signs of Aflatoxicosis are edema of the lower extremities, abdominal pain, and vomiting (Shepard 2003, Williams et al, 2004).

The first documented reports of aflatoxicosis in humans occurred in western India in 1974 where 397 persons were affected and 108 persons died in relation with ingestion of the molded maize (Krishnamaachari et al, 1975). Although on a global basis deaths from acute aflatoxin poisoning are rare, Kenya has experienced such episodes repeatedly for at least 25 years. Most recently in 2004, the largest and most severe outbreak of acute aflatoxicosis documented worldwide was

observed in Kenya in which 317 cases of acute aflatoxicosis were reported, resulting in 125 deaths (Case Fatality Rate = 39%). This epidemy was caused by ingestion of maize with aflatoxin concentrations of up to 4,400 μg/kg (Azziz et al, 2005)

Table 2 shows the response of different laboratory animals for a dose of aflatoxin B1 (i.e. the most potent carcinogen to cause acute toxicity)

Table 2. Acute toxicity of aflatoxin B1 expressed as a single oral dose LD_{50}

Species	**LD_{50} mg/kg body weight**
Rabbit	0.30
Pig	0.60
Cat	0.55
Dog	0.50-1.00
Chicken	6.30
Rat (male)	5.50-7.20
Rat (Female)	17.90
Mouse	9.00

Source: - Krishnamaachari et al, 1975).

Chronic toxicity is due to long term exposure of moderate to low aflatoxin concentration. The symptoms include decrease in growth rate, lowered milk or egg production, and immune-suppression in animals. Human liver cells are capable of metabolizing AFB1 to the biologically reactive 8,9-epoxide,which can form adducts with DNA (Moses. E.J and Neal G.E, 1985). DNA binding and inhibition of nucleic acid synthesis is the most common mechanism suggested for aflatoxin B1 action (Clifford and Rees, 1967; Stark, 1980). AFB1 is activated by cytochromes

P450 to AFB1-8,9-*exo*-epoxide and AFB1-8,9-*endo*-epoxide, but it is the *exo*-epoxide which binds to DNA to form the predominant 8,9-dihydro-8-(N7-guanyl)-9-hydroxy-AFB1 (AFB1–N7-Gua) adduct (Iyer *et al.*, 1994). AFB1–N7-Gua confers the mutagenic properties of the compound. The binding of the *exo*-epoxide to guanine reflects the geometry of intercalation between base pairs in the DNA helix; 5′ intercalation appears to facilitate adduct formation by positioning the epoxide for in-line nucleophilic reaction with the N7 guanine This intercalation epoxide causes a transversion at codon 249 in p53 gene in liver which may lead to hepatic carcinoma (Gopalakrishnan *et al.*, 1990; Kobertz *et al.*, 1997). Liver damage is apparent due to the yellow color that is characteristic of jaundice, and the gall bladder becomes swollen. Immuno-suppression is due to the reactivity of aflatoxins with T-cells, decrease in Vitamin K activities, and a decrease in phagocytic activity in macrophages. There is observed carcinogenecity, mainly related to aflatoxin B1. A numbers of studies have revealed an association between liver cancer incidence and the aflatoxin content of the diet. These studies have not established a cause and effect relationship, but rather suggested an association (Bommakanti et al, 2000)

Outbreaks of aflatoxic hepatitis due to chronic intake of aflatoxins in humans have been reported in India, Kenya, and Malaysia. Their effect on the health of organisms can be evaluated under two groups: biochemical and biological effects.

From biochemical point of view; they have effects on energy metabolism, carbohydrate and lipid metabolism, nucleic acid and protein metabolisms. On the biological point of view they are highly hepatocarcinogenic, teratogenic and mutagenic compounds (Zhang et al,2010).Major biochemical effects of mycotoxins involve the modification of normal metabolic and other vital processes. The mode of their action appears to be based primarily on their ability to interact with macromolecules, subcellular organelles and organs. Many of these aflatoxin induced effects may

be derived from their disruption of nucleic acid or protein synthesis. The effect of mycotoxins on carbohydrate metabolism is generally discerned as reduced hepatic glycogen and increased blood glucose levels. Aflatoxins decrease the liver glycogen level, inhibiting biosynthetic enzymes such as glycogen synthetase and by increasing the activity of enzymes metabolizing glycogen precursors, e.g. the NADP reducing enzyme glucose 6-phosphate dehydrogenase (Shankaran et al, 1970). Aflatoxins have been shown by Chou and Marth to affect lipid metabolism by increasing the cytosolic level of NADPH necessary for fatty acid synthesis in liver (Chou and Marth, 1975).

The risks associated with exposure to aflatoxins are enhanced by simultaneous exposure to hepatitis B and possibly hepatitis C viruses (Ramesh and Siruguri, 2003). Recently published results indicate that both being underweight and immune function are directly affected by aflatoxin exposure in developing countries. Studies in Benin and Togo, West Africa, have shown that aflatoxin exposure in infants, which increases at weaning, can lead directly to growth impairment and stunting (Gong et al, 2004).

Aflatoxin has long been linked to kwashiorkor, a disease usually considered a form of protein energy malnutrition, although some characteristics of the disease are known to be among the pathological effects caused by aflatoxins in animals.

Aflatoxins cause major damage to the liver DNA by producing various epoxides. Many serum proteins that are vital for the body's overall growth are produced in the liver, hence the destruction of liver cells leads to a decrease in the protein manufactured, ultimately ending in Kwashiorkor due to protein deficiency (Hendrickse, 1997). It has been suggested that either aflatoxins could play a causal role in the disease or children suffering from the disease are at greater risk to the hazards of dietary aflatoxin (Adhikari et al, 1994). Studies in Gambia and

Ghana have begun to elucidate the role of aflatoxin in immune suppression in human populations. Aflatoxin exposure was associated with reduced levels of secretory immunoglobulin A (IgA) in Gambian children (Turner et al, 2003). Changes in differential subset distributions and functional alterations of specific lymphocyte subsets have been correlated with aflatoxin exposure in Ghanaian adults and indicate that aflatoxins could cause impairment of human cellular immunity that could decrease resistance to infections (Jiang et al, 2005).

3.4.Occurrence of afaltoxins in red pepper

Red peppers are reported to be contaminated with moulds and their toxic metabolites (See Table 3) and *Aspergillus flavus* is the predominant mold on chilli samples in several cases. Poor harvesting practices, improper drying, handling, packaging, storage and transport of chillies facilitate fungal growth and thus results in the increased risk of AFs contamination (Christensen et al, 1967, Flannigan & Hui, 1976, Scott and Keneddy, 1973). In Ethiopia aflatoxin levels ranged from 250 to 525 µg kg-1 was found in ground red pepper in a preliminary survey made on aflatoxin contamination level of shiro and ground red pepper (Fufa and Urga, 1996).

Table 3. The occurrence of AFB1 in red pepper reported in previous studies

Country	Pepper variety	n	Positive (%)	Range	References
USA	Red pepper	12	9 (75.0%)	ND-30	Wood (1989)
Pakistan	Red pepper	176	117 (66.0%)	ND-25	Ahmad and Ahmed (1995)
Ethiopia	Ground red pepper	64	8(12.5 %)	250-525	Fufa and Urga (1996)
Turkey	Red pepper	34	8 (23.5%)	1.6-15	YÂldÂrÂm et al. (1997)
Australia	Chilli powder	26	8 (30.8%)	5-10	Klieber (2000)
Europa	*Capsicum* spp. (chilli)	17	1 (5.9%)	<2– 9.7	Food Safety Authority of
England	Chilli powder	28	8 (28.6%)	<0.2– 13.9	Food Safety and Inspection

ND: AFB1 levels were below minimum detection limit. (Source:-Aydin et al, 2007)

3.5. Red pepper production in Ethiopia

The diverse climatic conditions of Ethiopia are suitable for the production of different type of red peppers in different parts of the Country. Ethiopia is amongst the top 10 red pepper producing countries, India, China, Myanmar, Mexico, Vietnam, Peru, Pakistan, Ghana and Bangladesh.

In Ethiopia, red Peppers are widely cultivated in Mareko (SNNPR), Alaba (SNNPR), Ziway (East Shewa), Dembi Dollo (West Wellega), Todalle (Jima Route) and Gojam-Gonder. In 2001/02 the production and yield of red peppers was estimated at 780,000 qt and 13.87 qt/ha respectively (Central Statistical Agency of Ethiopia, 2010).

In 2009/2010 report of the Ethiopia Central statistical Agency on production of crops, 1,593,275 Quintals of red pepper was produced for the "Meher "season (September-December) (Central Statistical Agency of Ethiopia, 2010). The table below comprises the amount and area of production for the indicated season.

Table 4. Red-pepper production in Ethiopia for the year 2009/2010

S.No	Region	Amount of production(in quintals)
1	Tigray	32,326.13
2	Amhara	480,032.75
3	Oromia	575,353.06
4	Beinishangul-Gumuz	32,306.42
5	SNNP	472,186.89

Source: - Central Statistical Agency of Ethiopia, 2010

According to the agricultural bureau of Markeo woreda, the study location of this research, 2,100 tons of red pepper was planned to harvest during the harvest season of 2011/2012 and a

total of 1,440 tons of red pepper was actually harvested. The cause for the decrease of the produce was due to a disease called bacterial root rot.

3.6. Factors influencing the development of aspergilus and aflatoxin production

The factors implicated in the growth of the fungus belonging to the genus Aspergillus in foods are as much those relating to the environment in which they develop (pH, composition of the food or water activity) as to extrinsic factors: ambient humidity, storage temperature and microbial competition (Zinedine & Manes, 2009).

Although the conditions described vary slightly depending on the bibliographical source, some authors report that A. flavus or A. parasiticus grow in a temperature ranging between minimum 10-12 and maximum 42- 43 °C, and optimally between 32 and 33 °C. They can grow in a wide pH range (2.1 to 11.2), with optimal growth between 3.5 and 8. In terms of water activity (aw), the minimum values for growth are between 0.80 and 0.83, and the optimum is 0.99(Sweeney and Dobson, 1998). With regard to toxinogenesis, the temperature range in which toxins are produced is from 12 to 40 °C, with the optimum between 25 and 30 °C. In A. parasiticus the proportion of production of aflatoxins B compared to G is greater at high temperatures (International Commission on Microbiological Specifications for Food Toxigenic Fungi, 1996). They are produced in a pH range between 3.5 and 8, with an optimum pH of 6. In terms of water activity (aw), the minimum for production is 0.82 for A. flavus with an optimum of 0.99 (Cousin et al, 2005).

The production of aflatoxins also depends on the sources of carbohydrates and nitrogen, phosphates, lipoperoxides and trace elements (Luchese and Harrigan, 1993). As a result, this production is favored by an environment rich in carbohydrates, although some substrates rich in

fat and protein like peanuts also allow aflatoxin production (Altug et al, 1990). In some cases, the presence of other fungi may reduce aflatoxin synthesis (Wicklow et al, 1980).

3.7. Review of global regulations for aflatoxins in red pepper

Human exposure to aflatoxins is limited by regulations that prohibit the use of crops containing excess quantities of aflatoxins for foods and feeds (Bandyopadhyay, 2007). Aflatoxins are regulated in part per billion (ppb) ranges with the maximum allowable level varying with country and intended use of the commodity. The quantity permitted in U.S. foods and feeds ranges from 0.5 ppb to 20 ppb, depending on how the material will be used (Dohlman, 2004). The EU has set the limit for aflatoxin in foods destined for human consumption at 2 ppb (aflatoxin B1) and 4 ppb (total aflatoxins). It is important to note that these maximum tolerated levels vary greatly among countries, requiring harmonization to remove the extreme variability in standards (Wagacha and Muthomi, 2008). Figure 2 shows the variation on maximum tolerable limits among countries. As of 2014 no international standard for aflatoxins existed. Until 1996, JECFA had recommended that dietary aflatoxin be kept to an irreducible level. After this evaluation, there have been a number of attempts to establish standards for aflatoxins in food and feeds, but it has been exceedingly difficult to reach a consensus on maximum levels that should be included in these standards (Wu, 2004). Major impediments to consensus are the wide variation in contamination levels worldwide and in the relative ability of nations to reduce aflatoxin levels in cost-effective manner (Wilson 2007).

According to FAO, (2003) only 15 African countries had regulatory limits for aflatoxins as of 2003 (FAO, 2003). Ethiopia has not yet adopted such standards for mycotoxins including aflatoxins (Legesse, 2010).

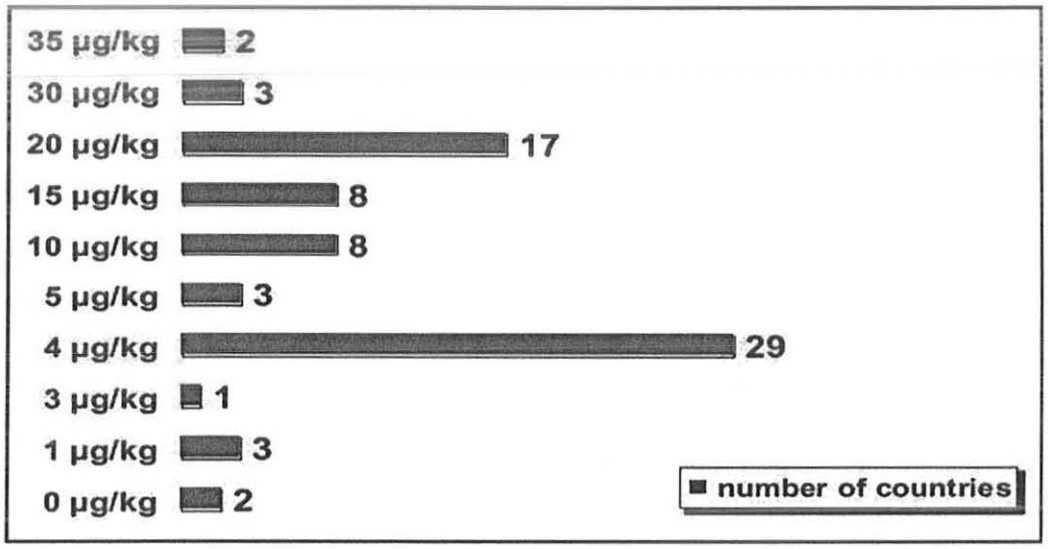

Figure 2.Worldwide limits for total aflatoxins in food
Source: - FAO, 2003

3.8.Detoxifications of aflatoxins

Since it is impossible to completely avoid some degree of aflatoxins contamination, a variety of strategies for their detoxification in foodstuffs have been proposed. These strategies have included physical methods of separation, thermal inactivation, irradiation, solvent extraction, adsorption from solution, microbial inactivation, chemical methods of inactivation and fermentation (Rosemary et al, 2007).

A wide range of chemicals have been tested for the ability to degrade and inactivates aflatoxins. Although a number of these chemicals can react to destroy aflatoxins effectively, most are impractical, too expensive or potentially unsafe because of the formation of toxic residues, or the perturbation of the nutrient content of the food.

Two chemical approaches that have received considerable attention are ammonization and reaction with sodium bisulphate (Huchchannanavar, 2011). Studies have shown that treatment of aflatoxin contaminated corn with ammonia is an effective detoxification approach. Ammonia

appears to produces hydrolysis of the lactone ring and chemical conversion of the parent compound to numerous products that exhibit greatly reduced toxicity. Similarly, sodium bisulphate reacts with aflatoxins to form water soluble degradation products (Sinha, 1998).

An alternative approach is to attempt to reduce the absorption of aflatoxins from contaminated feed in animals. This may be achieved by the addition of inorganic sorbent materials such as sodium calcium aluminosilicate (HSCAS) in the diet of animals. HSCAS tightly binds aflatoxins in the gastrointestinal tracts of animals, preventing their absorption into the body so that they are passed out unabsorbed in the feces. This results in a major reduction in the body burden (i.e. exposure) of the animals to the mycotoxin (Phillips et al, 1995).

Aflatoxins are quite stable compounds and survive relatively high temperatures with little degradation. Their heat stability is influenced by other factors, such as moisture level and pH, but heating or cooking processes cannot be relied upon to destroy aflatoxins (Lawley et al, 2012).

The stability of aflatoxins in foods means that control is best achieved by measures designed to prevent the contamination of crops in the field and during storage. Pre-harvest control of aflatoxins and effective control measure in post-harvest handling and storage is important in order to minimize the risk of aflatoxin exposure in humans (FAO, 2001).

3.9.Analytical techniques for determination of aflatoxins

There are several different methods of analysis available for aflatoxins detection, and one(s) chosen by a laboratory will vary depending on the cost and the intended use of the test. Some laboratories may use more than one method, depending upon its circumstances. Cost of aflatoxins analysis depend partly upon the method used to detect their presence. Some methods of aflatoxin analysis are able to determine the concentration of mycotoxins present (Quantitative), others cannot (Qualitative).Some laboratories may screen samples for the

presence of aflatoxins, using relatively fast, easy and cheap methods of analysis. If the analysis finds a aflatoxin to be present, then the sample is re-analyzed using another method that can confirm the presence of aflatoxins and determines the amount present. The most commonly used methods for the determination of aflatoxins are immunological and chromatographic methods (Gilbert & Anklam, 2002)

Chromatographic methods

Thin layer chromatography (TLC)

TLC is one of the simplest, fastest, easiest and least expensive of several chromatographic techniques used in qualitative and quantitative analysis to separate organic compounds. It is a liquid chromatography consists of a mobile phase (developing solvent) and a stationary phase; a plate or a strip coated with a form of silica gel. The two types of TLC are normal phase and reversed phase. Normal phase is the terminology used when the stationary phase is polar; for example silica gel and the mobile phase is an organic solvent or a mixture of organic solvents which is less polar than the stationary phase.

Reversed phase is the terminology used when the stationary phase is silica bonded with an organic substrate such as a long chain aliphatic acid like C-18 and the mobile phase is a mixture of water and organic solvent which is more polar than the stationary phase (Robards et al, 1994).

High performance liquid chromatography (HPLC)

The most common method used currently for aflatoxins analysis is HPLC methods using fluorescence detection. The fluorescence detector provides improved sensitivity as compared to previous technologies and enables pictogram quantification of all four aflatoxins. The principle behind HPLC is a test portion is extracted with a solvent solution (methanol/Acetonitrile/water).

The sample extract is filtered, diluted with water or phosphate buffered saline to a specified solvent concentration and a specified pH (7.2), and a portion is applied on an immunoaffinity column containing antibodies specific to retain the four major aflatoxins (B1, B2, G1, & G2). The aflatoxins are removed (eluted) from the immunoaffinity column with neat methanol (HPLC grade), and then quantified by reverse-phase high performance liquid chromatography (RP-HPLC) with post column derivatization (PCD) involving hydroxylation under UV light at 265 nm wave length or halogenations and then followed detection by fluorescence detector(Holcomb et al 1992).

4. Materials and Methods

4.1. Time and Place of the study

The study covers a total of 12 months (December, 2012 to January 2013).The study was conducted in two places of Ethiopia, namely Mareko and Addis Ababa. Mareko is one of the gurage zone woredas in the Southern Nations, Nationalities and Peoples' Regional state.

This woreda is named after the Mareko people. The woreda is well known among Ethiopians for its production of a favorable variety of red pepper. Mareko is bordered on the southwest by the Silt'e Zone, on the northwest by Meskane, and on the east by the Oromia Region. Based on the 2007 Census conducted by the CSA, the woreda has a total population of 64,512, of whom 32,730 are men and 31,782 women.

Addis Ababa is located at 9°1′48″ north of equator and 38°44′24″ east longitude with an area of 540 km2 and 2500 meter above sea level. The city was founded in 1886 and it has been serving as the capital city of Ethiopia since its foundation. According to the 2010 census estimate the city had a population of about 3 million (CSA, 2010). Its average temperature is 16 ^{0}c while its annual rainfall is 1221 millimeters.

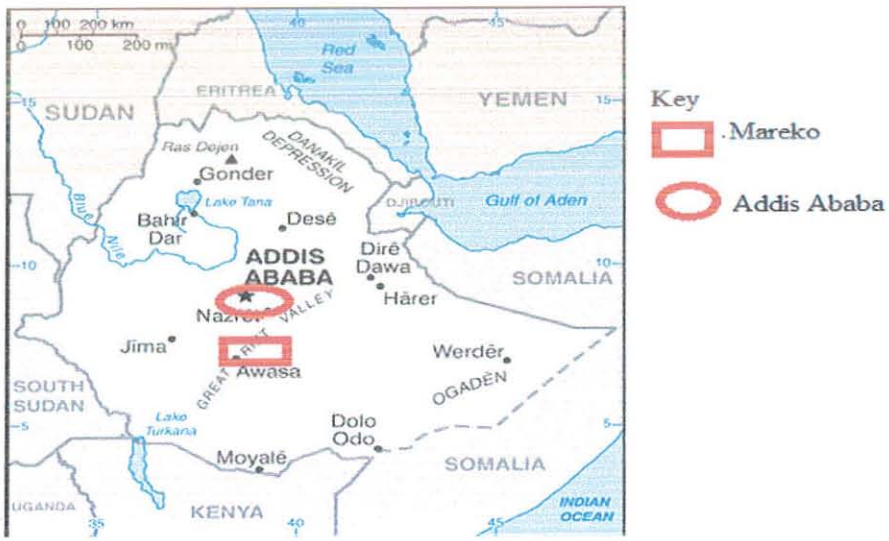

Figure 3. Map of study areas
(Source: Map produced by the U.S. Central Intelligence Agency)

4.2.Study design

To see the occurrence of aflatoxins in red pepper in relation with duration of storage and quality grade levels cross-sectional study design was followed.

4.3.Sampling and data collection

Samples stored for three months (n=10) were collected from farmers store at Mareko area, SNNP regional state of Ethiopia. The place and variety of red pepper was chosen based on the potential of red pepper production of the place, its uniquely identifiable variety and due to the variety's favorable quality in red pepper market of Ethiopia. The ten samples were collected from three randomly chosen localities of Mareko woreda (namely Dida Midore,Ale Mena and Gola Jardembeka) out of the eight major producer localities of Mareko woreda.

The three localities were chosen based on the information gathered from the agricultural bureau of the Mareko woreda for their production capacity of red pepper. i.e Out of the twenty six-

localities (kebeles) of Mareko woreda seventeen localities are producers of this product, but only eight localities are the major growers.

Samples of whole red pepper which were grown at Mareko and stored for six months in the wholesalers store in Addis Ababa (n=25) were collected from wholesalers store in Addis Ababa. The samples which were collected from the wholesalers store were classified by the merchants into three grades based on the quality of the red pepper as Grade-1,Grade-2 and Grade-3 and were clearly labeled on the sampling bag prior to transportation to laboratory. Grading the red pepper is mostly done by the merchants. The merchants give grading based on the condition of the red pepper. In which discolored, white and broken red peppers take the list grade (Grade 3) while red pepper with deep red color, high pungency and fleshly skin take the highest grade (Grade 1).Red pepper which will not fall in one of these two extremes take the medium grade (Grade 2).

Samples of grounded red pepper (n=10) were collected from ten different producers, locally called *Baltina houses* from Addis Ababa.

A questionnaire was used to gather information in relation to possible factors for aflatoxigenic fungi growth and subsequent aflatoxin contamination. The questionnaire was developed based on guidelines of good agricultural practices for red pepper production and questions in the questionnaire are depicted to look for storage conditions, packaging mode, duration of storage, drying conditions and other important practices (Annex 1 and Annex 2). Other relevant information was gathered by interview and observation.

Sampling was done based on the methods of sampling and analysis for the official control of the levels of mycotoxins in foodstuffs developed by the Commission of the European Communities (European Commission Regulation, 2006).

An aggregate sample of at least 5kg was prepared, grounded and thoroughly mixed and put in containers and stored frozen at -20 °c until analysis. Moisture content of the whole peppers stored for three months was determined within 48 hours of sampling. The moisture content for the whole red peppers stored for six months and for samples of grounded red peppers were determined within 24 hours after the samples were taken. Testing was carried out in Hilina Food laboratory accredited under ISO 17025 standard for the analysis of food proximate and aflatoxins in Food.

The following formula was used as a guide for the sampling of lots traded in individual packs, such as sacks, bags, retail packing etc.

$$\text{Sampling frequency (SF) } n = \frac{\text{(Weight of the lot} \times \text{Weight of the incremental sample)}}{\text{(Weight of the aggregate sample} \times \text{Weight of individual packing)}}$$

Weight: in Kg, Sampling frequency (SF): every n^{th} sack or bag from which an incremental sample was taken (decimal figures was rounded to the nearest whole number).

Table 5. Sampling frame used for collecting samples
(Source: - European Commission Regulation, 2006)

Lot weight (tonnes)	Number of incremental	Aggregate sample
≤ 0.01	5	0.5
> 0.01-≤ 0.1	10	1
> 0.1-≤ 0.2	15	1.5
> 0.2-≤ 0.5	20	2
> 0.5-≤ 1.0	30	3
> 1.0-≤ 2.0	40	4
> 2.0-≤ 5.0	60	6
> 5.0-≤ 10.0	80	8
> 10.0-≤ 15.0	100	10

4.4.Moisture determination

To determine the moisture content, AOAC method (AOAC, 2000) was used. Briefly, duplicates of 5.00 g of samples were placed in pre-washed and dried dish and then heated in oven at 105 o^c for 3.5 hour. They were cooled in desiccators and weighed until constant weight achieved.

4.5.Analytical procedure for aflatoxin determination

Aflatoxin was determined as described in AOAC (2005) using HPLC analyses JASCO HPLC (EX. 365, EM, 440nm). Chrompass chromatography soft ware program was applied for calculation LCTech C18 column (4.6 x 150 mm, 4μ) was used. Mobile phase: Acetonitrile, H2O, Methanol (15, 60, 25 v/v) was used for the anlaysis. The AFB1, AFB2, AFG1, and AFG2 mixed standards were obtained from Sigma-Aldrich (Ref A6636 – Batch No. 118K4094) the pure reference standards were stored in the dark at 4°C. Standard solutions of all four aflatoxins were prepared in methanol and stored in the dark at 4°C.

Sample preparation

From well mixed composite sample, 20g analytical sample were taken and prepared for the extraction.

Extraction

To 20g of analytical sample 2.0g of NaCl was added and extracted with 100 ml methanol/water (4/1) and 50 ml n-Hexene at high speed in a blender jar.

Clean up

The extract was passed through a plated filter and 14 ml of the filtered extract was diluted with 86 ml PBS-Puffer and tween 20 solutions (PH 7.2). After opening the immunoaffinity column, the

storage buffer was drained until the level reaches the upper frit and 50 ml of diluted extract was passed through the immunoafinity column.

The sample was left to drain through the column until no more sample in the column left and then the column get washed with 10 ml distilled water and residual water was removed by a gentle gas stream(i.e. nitrogen). The aflatoxin retained in the column was eluted with 2ml of methanol and collected in vial.

Derivitization

Since only the aflatoxins B2 and G2 readily fluorescence, aflatoxin B1 and G1 must be derivatized prior to detection. This was achieved photochemically by irradiation with UV light at 254nm with a photochemical reactor (LC Tech UVE, Germany).

HPLC determination

The eluted aflatoxin-methnol solution was directly measured by HPLC for its aflatoxin content using HPLC conditions as described in the method. Fluorescence detector setting (EX. 365, EM, 440nm).Column oven Temperature (39^0C), Injection volume (20µl) and Mobile phase composition: Acetonitrile , H2O , Methanol (15,60,25 v/v) was used for the anlaysis.

Analytical quality assurance

Samples were processed in batches of n=6–12 along with spiked sample and a blank. Samples were spiked with five concentration levels (5, 10, 20, 30 and50 µg/kg) with AFB1, AFG1, AFB2 and AFG2. The fortified samples were extracted and analyzed using the entire procedure. The batch gets repeated during cases where the recovery rate of the spikes becomes less than 70%.

Method performance

The accuracy and the precision of the method were determined by replicate analysis of samples containing known aflatoxin amounts. For that purpose test standards (prepared separately from the calibration standards) were used. The accuracy ranged from 96 to 111 % and the precision from 3.6 to 3.7%.The result showed good accuracy and precision compared with codex for acceptable accuracy at the 10 ppb level of 70–125% indicating the method was fit for the purpose.

The linearity of the methods was measured by calculating correlation coefficient r^2 of concentration versus peak area standard solutions of five different concentrations (5, 10, 20, 30, & 50 ppb). The result of correlation coefficient was determined from five point standard curve showed that 0.9755, 0.9974, 0.9946, and 0.9911 for B1, B2, G1, & G2 respectively. The result indicates the linearity was good enough for the analysis.

The specificity of the method was assessed by the visual inspection and comparison of chromatograms of blank samples and samples containing different amounts of aflatoxins. Because of the separation power of the chromatographic column and the selectivity of the fluorescence detector, no interfering peaks were observed near the retention times of the different aflatoxins in the blank samples. Also the AF peaks in the positive samples were symmetrical, proving the specificity of the method.

4.6.Analytical procedure for aflatoxin producing mycroflora isolation

Samples of red pepper samples from whole pepper stored for three months (FS003), whole red pepper stored for six months (WS017) and powdered red pepper sample (P006) was randomly chosen from the total samples gathered and used for the isolation of Aspergilus *flavus*.

10gm of red pepper sample was mixed with 90ml of buffered peptone water for the first dilution and from this dilution 1ml of sample was taken from each consecutive dilution to make the second and third dilutions respectively. 1ml of the homogenateed red pepper sample from each dilution was pippeted into marked duplicate petridishs containing 15ml of prepared potato dextrose agar (PDA). Samples in duplicate petri dishes then incubated inverted 25^0c for 5-7 days. Similar procedure was used repeatedly until a clear isolates of Aspergilus flavus found.

Uninoculated PDA medium incubated at 25^0c for 5-7 days was used as negative control. American public health association (APHA) 1976 method was used as a reference (American Public Health Association, 1976).

In order to see aflatoxin production of the isolates, spore suspension of *A. flavus* isolated from whole red pepper sample stored for three months (FS003), Whole red pepper sample stored for six months (WS017) and powdered red pepper sample (P006) respectively inoculated to fifty gram of blank red pepper sample in 500 ml Erlenmeyer flask. The cultures were then incubated at 25^0c for one week and 20 g of oven dried of each well mixed culture were then extracted for aflatoxins and aflatoxins were determined by HPLC.

4.7.Statistical analysis

Data obtained were analyzed statistically to calculate the level of significance of various parameters using analysis of variance technique by SPSS Software Package Version 16.0 and data were reported as mean. Data was first normalized to log(x+1) before a statistical calculation made. Student t-test and one way analysis of variance was used to see a difference between mean values. A probability level $p < 0.05$ was used to denote the statistically significant differences.

5. Results and Discussions

5.1. Post harvest handlings and occurrence of aflatoxin

Post harvest handlings and occurrence of aflatoxins at farmers level

Post harvest handlings that may predispose aflatoxin contamination such as drying on soil, not discarding peppers with problems before drying, not having a mechanism for protecting the peppers from rain and pests while drying, using packaging not suitable for aeration and not storing the red pepper in a place which is prevented from access by insects, rodents and other animals were found common in the present study based on the information gathered by a questionnaire prepared to collect information about post harvest management practices of the farmers (see Annex 1). As illustrated in Table 6, the farmers dry the whole red peppers to a moisture content level ranging from 7.52-13.47% with a mean moisture content of 9.16%. According to good agricultural practices for red pepper the moisture content must be reduced to 10% because of the reason that moisture content above this range could support mould growth and aflatoxins contamination during storage (Government of India Ministry of Agriculture, 2009). Based on this fact, in this research 80% of the samples were found dried to a recommended level (<10%). (See Table 6) However, as shown in Annex 1, 90% of the study population had found practicing drying red pepper intact with soil ground. This practice could aggravate aflatoxin contamination as the red pepper pods could pick dirt and more aflatoxin producing fungi from the soil during drying. Only 10% of respondents in the study population had practiced drying on yards, clean and dry polyethylene sheets or any other means to prevent contamination with soil.

All of the respondents in the study population had practiced the rest of the abovementioned post harvest handlings that could predispose aflatoxin contamination. All of the respondents hadn't practiced sprinkling water to the red pepper pods before selling the produce to merchants. The

farmers (70% of the respondents) mostly stored the red pepper in polyethylene bag for not more than four months as the produce is a cash crop (See Annex 1). Plate 1 show the storage condition in one of the farmers' store which can serve as an example as it is mostly found in many farmers store.

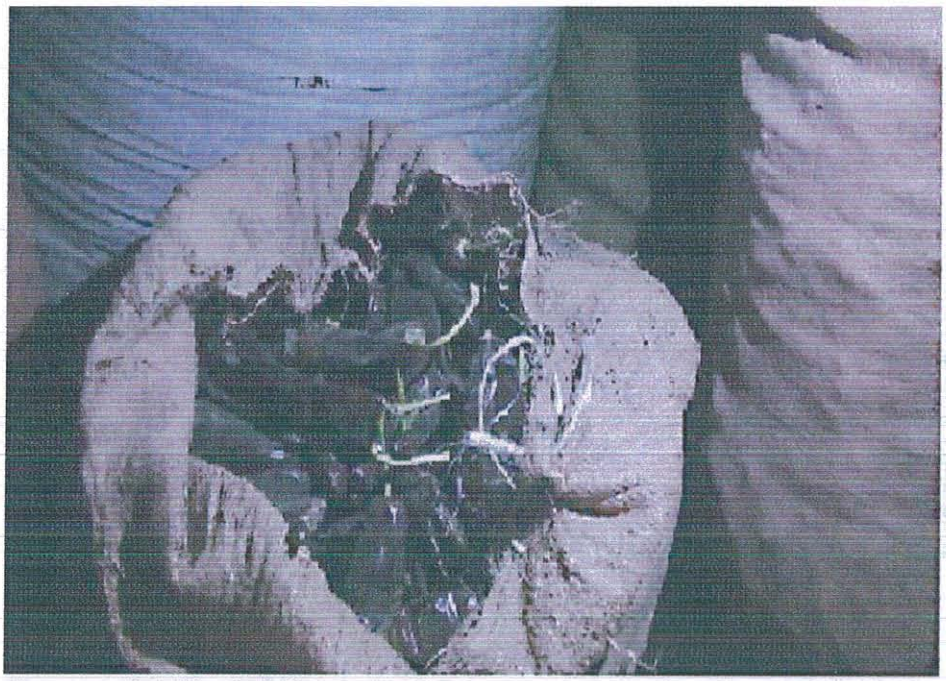

Plate 1. Whole red pepper stored in one of farmers store at mareko, Dida midore kebele

As shown in Table 6, from the total red pepper samples analyzed, aflatoxin was detected in 70% of the samples with the range of 7.45µg/kg to 86.33µg/kg with a mean value of 19.56 µg/kg. Out of the total samples analyzed 50% of the samples showed aflatoxin contamination above the European Commision maximum limit for total aflatoxins (10 µg/kg). The mean level of aflatoxin B1(7.02 µg/kg) and G1(10.90 µg/kg) was much higher than the mean level of Aflatoxin B2 (0.80 µg/kg) and Aflatoxin G2(0.84µg/kg) ($P < 0.05$).The most potent carcinogenic agents Aflatoxin B1 and G1 were detected in 70% of samples while the less potent ones Aflatoxins B2 and G2 were detected in 50% of samples analyzed (See Table 6).

Table 6. Aflatoxin and moisture content of whole red pepper stored at farmers store

S.No	Place of Sampling	AFG2 (µg/kg)	AFG1 µg/kg)	AFB2 (µg/kg)	AFB1 µg/kg)	Total Aflatoxin (µg/kg)	Moisture (%)
1	Dida Midore	ND	ND	ND	ND	ND	8.2
2	Dida Midore	ND	ND	ND	ND	ND	8.47
3	Dida Midore	0.58	3.58	1.03	5.07	10.26	7.52
4	Ale Mena	0.66	50.43	2.22	33.02	86.33	13.74
5	Ale Mena	1.23	2.95	1.18	2.09	7.45	8.03
6	Ale Mena	ND	7.43	ND	3.52	10.95	8.76
7	Gola Jardembeka	3.58	4.45	2.41	3.08	13.52	9.00
8	Gola Jardembeka	ND	6.03	ND	3.41	9.44	7.84
9	Gola Jardembeka	2.36	34.13	1.18	20.00	57.67	10.98
10	Gola Jardembeka	ND	ND	ND	ND	ND	9.03
Mean		**0.84**	**10.90**	**0.80**	**7.02**	**19.56**	**9.16**

ND: Not Detected

Post harvest handlings and occurrence of aflatoxins at wholesalers level

Based on the questionnaire prepared to gather information about storing conditions and other practices of the wholesalers that may predispose aflatoxin contamination; storing for more than six months, storing in a store which doesn't have prevention from access by insects or rodents, sprinkling water on red pepper pods and storing in a package which is not suitable for aeration were found common in the present study (See Annex 2).

Most of the respondents in the study population had practiced sprinkling water to the red pepper pods. According to the respondents, they add water to red pepper pods; to add weight to the pods, to prevent the stalks of the pods from breakage and to give a view of fleshy skin for the pod were among the main reasons. This is in agreement with the high moisture content found at this level in which the moisture content of samples were found ranging from 18.74-25.03% with a mean moisture content of 21.51% (see Table 7). The mean moisture content for samples stored at wholesalers store (21.51%) was found two times greater than the mean moisture for samples stored at farmers store. The practice of sprinkling water by the merchants on red pepper pods was taken as a reason for the observed differences in this study in mean moisture content of samples stored at farmers and wholesalers store respectively.

As shown in Table 7, from the total red pepper samples analyzed, aflatoxin was detected in all samples. The amount of aflatoxin found ranges from 1.19 μg/kg to 265.51 μg/kg with mean level of 78.83μg/kg. Out of the total samples analyzed 90% showed aflatoxin contamination above the Europian Commission permissible limit for total aflatoxins (10 μg/kg). The mean level of aflatoxin B1 (37.37 μg/kg) and G1 (37.30 μg/kg) was much higher than the mean level of Aflatoxin B2 (2.86μg/kg) and Aflatoxin G2 (1.30μg/kg).

The most potent carcinogenic agents Aflatoxin B1 and G1 were detected in 100% and 90% of samples analyzed respectively while the less potent ones Aflatoxins B2 and G2 were detcted in 80% and 70% of samples analyzed respectively.

Table 7. Aflatoxin and moisture content of Whole red pepper stored at wholesalers store

S.No	AFG2 (µg/kg)	AFG1 (µg/kg)	AFB2 (µg/kg)	AFB1 (µg/kg)	Total Aflatoxin (µg/kg)	Moisture (%)
1	1.44	38.02	1.25	15.07	55.78	21.88
2	ND	5.45	ND	8.02	13.47	18.74
3	ND	13.33	0.79	8.25	22.37	18.82
4	3.71	146.34	8.5	106.96	265.51	22.11
5	ND	ND	ND	1.19	1.19	21.92
6	0.54	15.77	2.38	38.63	57.32	21.78
7	0.61	17.74	1.32	26.8	46.47	21.14
8	4.82	107.94	9.5	102.66	224.92	25.03
9	0.97	13.56	3.45	34.67	52.65	21.92
10	0.92	14.87	1.38	31.45	48.62	21.78
Mean	**1.30**	**37.30**	**2.86**	**37.37**	**78.83**	**21.51**

ND: Not Detected

5.2. Aflatoxin and moisture content in relation to duration of storage

As shown in Table 8, the total aflatoxin content shows a significant difference ($P<0.05$) across storage duration such that samples stored for three months had 19.56 µg/kg mean aflatoxin content while 78.83 µg/kg for samples stored for six months. The six month stored red pepper sample shows a fourfold greater in mean total aflatoxin content compared to a red pepper sample stored for three months. Of the four aflatoxins only aflatoxin B1 shows a statistical significant difference ($P<0.05$) between red pepper samples stored for three months and six months. The mean aflatoxin B1 content stored for six months was 5 fold greater(7.02 µg/kg) compared with that of red pepper sample stored for three months (37.37µg/kg) .The other three aflatoxins (i.e. Aflatoxin G1,Aflatoxin G2 and aflatoxin B2) shows no significant difference between red

pepper samples stored for three months and six months. The mean aflatoxin G1 content for the six month stored samples (37.30 µg/kg) was three times greater than sample stored for six months.

Equally to the fact that the longer the storage time, the greater the possibility of building up environmental conditions conducive to aflatoxigenic mould proliferation and subsequent aflatoxin production, the practice of frequent sprinkling of water on whole red pepper pods for different reasons by the wholesalers could be a reason for the observed aflatoxin content difference in this research between whole red pepper stored for three months and six months.

Table 8. Mean aflatoxin and moisture content of whole red pepper stored for 3and 6 months

Duration of storage	**Mean levels of aflatoxin and moisture content**				
	AFG2 (µg/kg)	**AFG1 (µg/kg)**	**AFB2 (µg/kg)**	**AFB1 (µg/kg)**	**Total Aflatoxin (µg/kg)**
3 months	0.84^{a}	10.90^{a}	0.80^{a}	7.02^{a}	19.56^{a}
	ND-3.58	ND-50.43	ND-2.41	ND-33.02	ND-86.33
6 months	1.30^{a}	37.30^{a}	2.86^{a}	37.37^{b}	78.83^{b}
	ND-4.82	ND-146.34	ND-3.45	1.19-106.96	1.19-265.51

ND-Not Detected

At each level of parameter, the mean with different superscript in the same column are significantly different at $P<0.05$ level.

Figure 4 shows the comparison of aflatoxin and moisture content in relation with duration of storage.

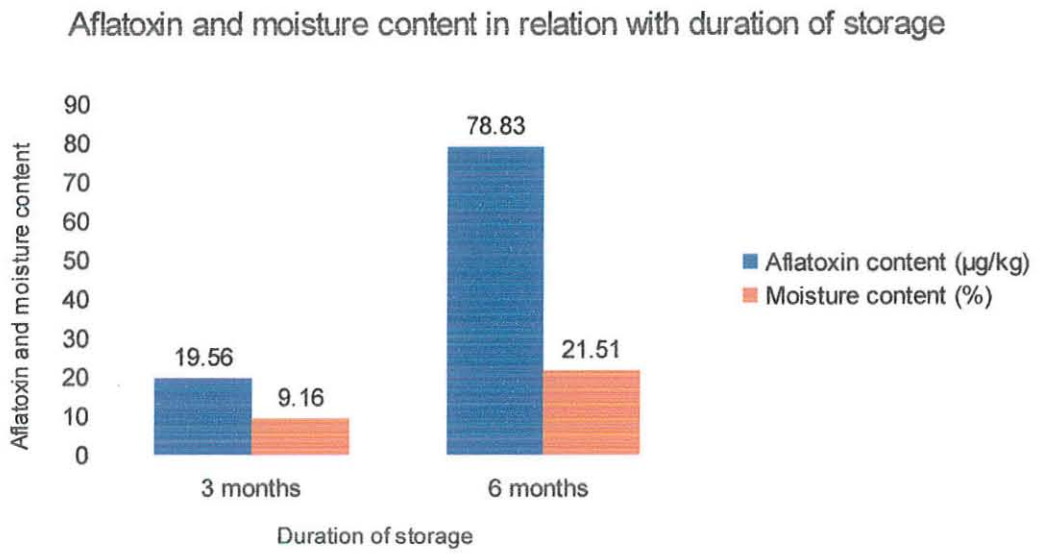

Figure 4.Aflatoxin and moisture content in relation with duration of storage

5.3.Aflatoxin and moisture content in relation to quality grade levels of red pepper

As illustrated in Table 9, the mean moisture content in all the grade levels were above the maximum recommended level (≤10%) The moisture content for Grade 1 ranges from 18.74-25.03% with a mean moisture content of 21.51%. Moisture content for Grade 2 ranges from 13.35-25.87% with a mean moisture content of 17.64. Moisture content for Grade 3 ranges from 16.09-23.29% with a mean moisture content of 17.83%.

However, the mean moisture was not statistically significant ($P<0.05$) to show a difference across the three grade levels in this reserach.

Aflatoxin was detected in all samples classified as Grade 1, Grade 2 and Grade3.The highest aflatoxin content was found in the least grade level (Grade 3) with a concentration of 399.19μg/kg. The least aflatoxin content was found in the high quality grade level (Grade 1) with a concentration of 1.19μg/kg. Aflatoxin content for Grade 1 ranges from 1.19-265.51μg/kg with a mean concentration of 78.83μg/kg.

In Grade 2 red pepper sample, aflatoxin was found ranging from 28.42-250.13μg/kg with a mean concentration of 160.11μg/kg. Aflatoxin content for Grade 3 red pepper ranges from 70.80-399.19μg/kg with a mean concentration of 312.33μg/kg. The mean aflatoxin content found was above the European Commission maximum limit (10μg/kg) in all the three Grade levels.

The percentage of samples contaminated with aflatoxin above the EU limit was found 80% for Grade 1 while 100% for Grade 2 and Grade3 respectively. The mean aflatoxin content found was approximately 7 times (for Grade1),16 times (for Grade2) and 30 times (for Grade 3) greater than the EU permissible limit(10μg/kg).

As indicated in Table 7, the result shows no significant difference ($P<0.05$) in aflatoxin G2 and B2 content between the three grade levels. However, Aflatoxin B1 content shows a significant difference ($P<0.05$) between Grade 2 and 3 while aflatoxin B1 content between Grade 1 and 3 shows no significant difference ($P<0.05$). A very significant difference ($P<0.05$) was shown in Aflatoxin G1 and total aflatoxin content between Grade 1, Grade 2 and Grade 3 whole red pepper samples.

Table 9. Mean aflatoxin and moisture content of the three grade levels of whole red pepper

Grade	Mean levels of aflatoxin and moisture content				
	AFG2 (µg/kg)	AFG1 (µg/kg)	AFB2 (µg/kg)	AFB1 (µg/kg)	Total Aflatoxin (µg/kg)
Grade 1	1.86[a]	41.45[a]	3.57[a]	37.37a	78.83[a]
	ND-4.82	ND-146.34	ND-3.45	1.19-106.96	1.19-265.51
Grade 2	1.92[a]	104.65[b]	3.71[a]	49.82[a]	160.11[b]
	ND-3.19	1.31-226.64	1.46-9.74	20.90-118.52	28.42-250.13
Grade 3	0.80[a]	168.37[c]	4.14[a]	139.02[b]	312.33[c]
	ND-1.67	35.49-215.24	2.83-6.00	30.86-179.54	70.80-399.19

At each level of parameter, the mean with different superscript in the same column are significantly different at $P<0.05$ level.

As shown in Table 9, aflatoxin content greatly varies between the three Grade levels. The research result is comparable with Reddy et al (Reddy et al 2001) in which red pepper with Grade 1 shows the lowest contamination while red pepper sample with Grade 3 showing the highest contamination. But the maximum aflatoxin found in Grade 3 red pepper by Reddy et al 2001 (966.7µg/kg) was much higher than the maximum aflatoxin found in the same Grade level (312.33µg/kg) in this research.

The reason for the variability of aflatoxin content between the three grade levels could be because of the relation between possible causes of aflatoxin contamination and the criteria set by the merchants for Grading. The criteria for setting the grading by the merchants which mainly depend on moldiness (rotten odor), discoloration (white, grayish, or diseased-looking spots) and texture of the pod (soft, mushy) could have an impact on aflatoxin producing mycroflaora

growth and subsequent aflatoxin contamination. Plate 2 shows the three Grade levels of red peppers sold at merchants ware house.

Grade 1 Grade 2 Grade 3

Plate 2. Pictures of the three red pepper Grade levels

5.4.Occurrence of afaltoxin in red pepper powder from retail stores

As shown in Table 10, the result of moisture determination showed that the moisture content of samples was ranged between 9.08 % and 13.24%. The mean level of moisture content was found to be 11.85%. From the total red pepper samples analyzed for aflatoxins presence, aflatoxin was detected in all samples. The amount of aflatoxin found ranges from 18.37µg/kg to 158.87µg/kg. The mean level of aflatoxin content was 66.34µg/kg. All of the samples analyzed showed aflatoxin contamination above the European Commission maximum limit for total aflatoxins in ground red pepper (10 µg/kg.) The mean level of aflatoxin B1 (29.60 µg/kg) and G1 (35.28 µg/kg) was much higher than the mean level of Aflatoxin B2 (1.17µg/kg) and Aflatoxin G2 (0.28µg/kg).

Incidence of aflatoxin contamination on the examined red pepper samples appear to be higher than those reported on spices in other similar researches (Fufa and Urga,1996 ,Ahmad I & Ahmad S,1995)The result also shows a very high contamination of aflatoxins above the permissible limit compared to other researches.

In this research 100% of the samples shows aflatoxin contamination above the European Commission permissible limit while same researches in India 9%, Turkey 18% (Reddy et al. 2001, A.Aydin et al, 2007) of red pepper powders sold in supermarkets contained non-permissible aflatoxin levels. However, the maximum aflaoxin content found in this reserch (158.87µg/kg) was below the maximum amount reported by Fufa and Urga (1996) (525µg/kg). The table below shows level of aflatoxin and moisture content of red pepper powder produced by different manufacturers locally called *Baltina* houses.

Table 10. Aflatoxin and moisture content of grounded red pepper

S.No	AFG2 (µg/kg)	AFG1 (µg/kg)	AFB2 (µg/kg)	AFB1 (µg/kg)	Total Aflatoxin (µg/kg)	Moisture (%)
1	ND	26.91	0.84	18.32	46.07	12.01
2	0.50	52.88	1.65	37.03	92.06	13.04
3	ND	9.98	ND	8.39	18.37	9.08
4	0.86	51.09	2.47	44.12	98.54	10.97
5	ND	21.68	0.95	19.21	41.84	10.72
6	ND	11.54	ND	6.44	17.98	12.07
7	ND	25.52	0.57	15.8	41.89	11.32
8	0.80	70.63	3.18	84.26	158.87	12.86
9	ND	14.24	ND	10.87	25.11	13.23
10	0.66	68.37	2.06	51.59	122.68	13.24
Mean	**0.28**	**35.28**	**1.17**	**29.60**	**66.34**	**11.85**

ND-Not Detected

5.5. Isolation of aspergilus from different red pepper samples

To better isolate the Aspergilus species from the red pepper samples, simple growth analyses made on agar plates were carried out. Several fungal species became obvious from the growth

media after seven days of incubation in all the three dilutions. Dilution in 10^{-1} and10^{-2} level shows over growth of different fungi species in all the red pepper samples (i.e Whole red pepper samples stored for three months; FS003, Whole red pepper samples stored for six months; WS017 and powdred red pepper sample P006) and was not used in further isolation of Aspergillus *flavus*. As a result samples with dilution factor of 10^{-3} were used for further isolation of Aspergilus *flavus* in all the three samples. From the seven days onwards, infected samples displayed fungal species, which were obvious enough for isolation of Aspergilus *flavus*.

Identification of aflatoxin producing isolate

The identification of Aspergillus was based on macroscopic analysis. The macroscopic identification involves observing the cultural characteristics of Aspergillus in the petri dishes; in this research color of colonies was used as a way of identifying the species. *Aspergillus flavus* is clearly differentiated by producing a characteristic orange pigment in a plate using the method described in Pitt *et al* (1983). (See Plate 3)

Plate 3. Some of the isolates of A. flavus

Isolated A. flavus from the three samples (i.e whole red pepper sample stored for three months

(FS003), Whole red pepper sample stored for six months (WS017) and powdered red pepper sample (P006)) were found capable for production of aflatoxins. The total aflatoxin content was found to be 91μg/kg, 113μg/kg and 85 μg/kg for *A. flavus* isolates from whole red pepper sample stored for three months, Whole red pepper sample stored for six months and powdered red pepper sample respectively. The presence of the Aspergillus *flavus* and potential of the isolate from the powdered red pepper sample to produce aflatoxin could pose a risk to consumers as the grounded red pepper powder could be stored further at consumer level

6. Conclusions and Recommendations

In this study storage duration of whole red pepper for three months shows low ($P<0.05$) aflatoxin contamination compared to red pepper samples stored for six months.The mean aflatoxin content increases from 19.56 μg/kg to 78.83μg/kg within three months of storage after harvest.

The two fold increase in average moisture content (i.e 9.16% for 3 months and 21.51% for 6 months) produces a favourable environment for mycological growth and subsequent formation of high level of aflatoxins.The practice of sprinkling water on red pepper pods by the merchants to add weight to the pods, to prevent the stalks of the pods from breakage and to give a view of fleshy skin for the pod was observed as a main reason for the high aflatoxin contamination of red pepper samples stored for six months at merchants ware house.

Of the total samples with 3 months of storage duration 50% of the samples were below the European Commission (EU) permissible limit (10μg/kg) while only 10% of the samples stored for 6 months were below the European Commission (EU) permissible limit (10μg/kg).

In this research, the amount of aflatoxin contamination shows an increase when the quality of the red pepper gets worse. The mean aflatoxin content increases from 78.83 μg/kg for Grade1 to 312.33 μg/kg for Grade 3. The mean aflatoxin content found was approximately 7 times (for

Grade1), 16 times (for Grade2) and 30 times (for Grade 3) greater than the European Commission (EU) permissible limit (10μg/kg)

The amount of aflatoxin in red pepper powder traded in retail stores ranges from 18.37μg/kg to 158.87μg/kg with a mean level of aflatoxin content 66.34μg/kg. In this research 100% of the grounded red pepper samples surpass the European Commission (EU) permissible limit (10μg/kg).

The result in this research indicated that all the isolated A. flavus from the red pepper samples were capable for production of aflatoxins.

In order to reduce aflatoxin contamination of red peppers; sufficient hygiene conditions during drying, transport and storage stages should be maintained so as to reduce microbiological and mycological growth which could result in the formation of aflatoxins. Moreover, the practices of sprinkling water on whole red pepper pods by merchants, which was observed in this research as possible reason for high level of aflatoxins, should be taken in to account when dealing with aflatoxin management for red pepper in Ethiopia. The use of high quality red peppers should be practiced whenever possible as Grade 1 red peppers shows relatively low aflatoxin contamination compared to red pepper traded as Grade 2 and Grade 3.

In general, aflatoxin management in red pepper which involve farmers, traders (wholesalers and retailers), processors, and policy makers especially those involved in inspection and regulatory control should be established for effective management of the contamination.

7. References

1. Abate D. and Gashe, B (1985). Prevalence of *Aspergillus flavus* in Ethiopian cereal grains: A preliminary survey. *Ethiopian Medical Journal* ***23***, 143-148.
2. Adhikari, M, Ramjee, G and Berjak, P (1994). Aflatoxin, kwashiorkor, and morbidity. *Journal of Natural toxins.*
3. Ahmad, IF and Ahmed, SK (1995). Contamination of red chili with Aflatoxin B1 in Pakistan. *Journal of Mycotoxin Research* ***11,*** 21–24
4. Altug, T., Yousef, A., & Marth, E. (1990). Degradation of aflatoxin B1 in dried figs by sodium bisulfite with or without heat, ultraviolet energy or hydrogen peroxide. *Journal of Food Protection (USA)* 53,581-582
5. American Public Health Association (1976). *Compendium of Methods for the Microbiological Examination of Foods.* American Public Health Association Inc. Washington DC
6. Andrallos, P. J., A. C. Beckwith, and R. M. Eppley (1967). Photochemical changes of aflatoxin B1. *Journal of Association of Analytical Chemists* ***50,*** 346-350
7. Asao, T., Buchi, G., Abdel-Kader, M., Chang, S., Wick, E., and Wogan, G. (1963). Aflatoxins B and G. *Journal of American Chemical Society* ***85,*** 1706-1707.
8. Ayalew, A., Fehrmann, H., Lepschy, J., Beck, R., & Abate, D. (2006). Natural occurrence of mycotoxins in staple cereals from Ethiopia. *Journal of Mycopathologia*, *162*(1), 57-63.
9. Aydin A., Emin M, Baokaya R, Ciftcioglu G. (2007). A Determination of Afatoxin B1 levels in powdered red pepper. *Journal of Food Control* ***18.***

10. Azziz-Baumgartner E., Lindblade K..,Gieseker K., Schurz Rogers H., Kieszak S., Njapau H., Schleicher R., McCoy L.F., Misore, A., DeCock, K., Rubin, C., Slutsker, L. and the Aflatoxin Investigative Group (2005). Case-control study of an acute aflatoxicosis outbreak – Kenya-2004. *Journal of Environmental Health Perspectives* ***113***, 1779-1783.

11. Bennett, J.W. and Klich, M. (2003). Mycotoxins. *Clinical Microbiology Reviews* ***16***, 497-516

12. Besrat, A. and P. Gebre(1981). A preliminary study of aflatoxin content of selected Ethiopian foods. *Ethiopian Medical Journal* ***19***,47-52.

13. Binder, E., Tan, L., Chin, L., Handl, J., Richard, J. (2007). Worldwide occurrence of mycotoxins in commodities feeds and feed ingredients. *Journal of Animal Feed Science and Technology* ***137(3)***.

14. Bandyopadhyay R., Williams J., and Ojiambo P. (2007). Social and political challenges in the regulation of mycotoxin contamination. *Journal of Phytopathology* ***97***,148.

15. Central Statistical Agency of Ethiopia (2010). *Agricultural sample survey 2009/2010: Area and production of crops* .Retrieved from the Central Statistical Agency of Ethiopia website http://www.csa.gov.et

16. Chala A.,Mohammed A,Ayalew A.Skinnes H.(2012).Natural occurrence of aflatoxins in groundnut (*Arachis hypogaea L.*) from eastern Ethiopia. *Journal of Food Control* ***30***,602-605

17. Chou, C., & Marth, E. (1975). Incorporation of (2-14C) acetate into lipids of mink (Mustela vison) liver and intestine during in vitro and in vivo treatment with aflatoxin B1. *Journal of Applied microbiology*, *30*(6), 946-950.

18. Christensen C.M., Fause H.A., Nelson G.H., Fern Band Mirocha C.J, 1967. Microflora of black and red pepper. *Journal of Applied microbiology, 15,622*–626._

19. Clifford, J., & Rees, K. (1967). The interaction of aflatoxins with purines and purine nucleosides. *Journal of Biochemical, 103*, 467-471.

20. Cousin M., Riley R. and Pestka .J. (2005). Food borne mycotoxins. *Academic Press*, 163-273.

21. Dohlman, E. (2004). Mycotoxin Regulations: Implications for International Agricultural Trade. *United States Department of Agriculture, Economic Research Service, Agricultural Information Bulletins No. 33623.*

22. Europian Commission Regulation (EC) No 401 (2006). Methods of sampling and analysis for the official control of the levels of mycotoxins in foodstuffs. *Official Journal of the European Union.*

23. FAO (2001).*Manual on the application of the HACCP system in Mycotoxin prevention and control.* FAO Food and Nutrition paper 73.Retrieved from the Food and Agriculture Organization of the United Nations website: http://www.fao.org

24. FAO (2003). *Worldwide regulations for mycotoxins in food and feed.* FAO Food and Nutrition paper 81.Retrieved from the Food and Agriculture Organization of the United Nations website: http://www.fao.org

25. Flannigan B. and Hui S. (1976). The occurrence of aflatoxin producing strains of *Aspergillus flavus* in the mold flora of spices. *Journal of Applied Bacteriology, 41,*411–418.

26. Fufa, H. and Urga K. (1996). Screening of aflatoxins in Shiro and ground red pepper in Addis Ababa. *Ethiopian Medical Journal. **34(4)*** 243-249

27. Fufa, H. and K. Urga (2001). Survey of Aflatoxin Contamination in Ethiopia. *Ethiopian Journal of Health Sciences **11(1)*** ,17-25.

28. Geyid, A. and Maru A. (1987). *A survey of aflatoxin contents in maize, sorghum and teff samples. Ethiopian Journal of Health Development. **2**,*59-70.

29. Gilbert, J., & Anklam, E. (2002). Validation of analytical methods for determining mycotoxins in foodstuffs. *Journal of Trends in Analytical Chemistry*, ***21*** **(6)**, 468-486.

30. Gong, Y., Hounsa, A., Egal, S., Turner, P., Sutcliffe, A., Hall, A. & Wild, C. (2004). Post weaning exposure to aflatoxin results in impaired child growth: a longitudinal study in Benin, West Africa. *Journal of Environmental Health Perspectives*, ***112*****(13)**, 1334.

31. Gopalakrishnan,S., Harris,T. and Stone, M. (1990) Intercalation of aflatoxin B1 in two oligodeoxynucleotide adducts: comparative 1H NMR analysis of d(ATCAFBGAT). d(ATCGAT) and d(ATAFBGCAT). *Journal of Biochemistry, 29,* 10438–10448

32. Government of India Ministry Of Agriculture (2009) .*Post harvest profile of chilli.*Retrieved from the Government Of India Ministry Of Agriculture,Department Of Agriculture & Cooperation website http://www.agmarknet.nic.in

33. Holcomb, M., Wilson, D., Trucksess, M. & Thompson J. (1992). Determination of aflatoxins in food products by chromatography. *Journal of Chromatography*, ***624*****(1)**, 341-352.

34. Hendrickse, R. (1997). Of sick turkeys, kwashiorkor, malaria, perinatal mortality, heroin addicts and food poisoning: Research on the influence of aflatoxins on child health in the tropics. *Journals of Tropical Medicine & Parasitology, 91(7),*787–793

35. Huchchannanavar, S., & Balol, G. (2011). Aflatoxins in food chain and their management. *Journal of Current Biotica*, *5*(2), 250-263.

36. IARC (1993). IARC Monographs on the Evaluation of Carcinogenic Risks to Humans: Some Naturally Occurring Substances: Food Items and Constitutents, Heterocyclic Armonatic Amines and Mycotoxins, *IARC Scientific Publications* No. 56. IARC, Lyon.

37. International Commission on Microbiological Specifications for Food. Toxigenic fungi (ICMSF) (1996). *Aspergillus. Microorganism in foods :Characteristics of Food Pathogens.* Academic Press, London.

38. Iqbal, S. Z., Asi, M. R., Zuber, M., Akhtar, J., & Jawwad Saif, M. (2013). Natural occurrence of aflatoxins and ochratoxin A in commercial chilli and chilli sauce samples. *Journal of Food Control, 30*(2), 621-625.

39. Iyer, R., Voehler,M. and Harris,T.. (1994) Adenine adduct of aflatoxin B1 epoxide. *Journal of American Chemical Society, 116,* 8863–8869

40. Jiang, Yi Jolly, Pauline E Ellis, William O Wang, Jia-Sheng Phillips, Timothy D Williams, Jonathan H (2005). Aflatoxin B1 albumin adduct levels and cellular immune status in Ghanaians. *Journal of International Immunology,* ***17(6),*** 807-814

41. Kobertz, W., Wang, D., Wogan, G. and Essigmann, J. (1997). An intercalation inhibitor altering the target selectivity of DNA damaging agents: synthesis of site-specific aflatoxin B1 adducts in a p53 mutational hotspot. *Journal of Proceedings of the National Academy of Sciences.* 94, 9579

42. Kumeda, Y., & Asao, T. (2001). Heteroduplex Panel Analysis, a Novel Method for Genetic Identification of Aspergillus Section Flavi Strains. *Journal of Applied and environmental microbiology, 67*(9), 4084-4090.

43. Krishnamachari, K.A., Nagaarajan, V., Bhat, R.V., Tilak, T.B. (1975). Hepatitis due to aflatoxicosis—an outbreak inWestern India. *Lancet* ***305***: 1061–1063.

44. Legess E, and Abate D (2010). Aflatoxin Content of Peanut (*Arachis hypogaea*) in Relation to Shelling and Storage Practices of Ethiopian Farmers, Thesis, Addis Ababa University.

45. Lawley, R., Curtis, L., & Davis, J. (2012). *The food safety hazard guidebook.* Royal Society of Chemistry.

46. Luchese, R.H. and Harrigan, W. (1993). Biosynthesis of aflatoxin - the role of nutritional factors. *Journal of Applied Microbiology, 74*,5-14

47. Moses.E.J and Neal G.E, (1985). The metabolism of aflatoxin B1 by human liver. *Journal of Biochemical Pharmacology, 34, 3193*-3197.

48. Nissen, N., & Martin, P. (2002). Hepatocellular carcinoma: the high-risk patient. *Journal of clinical gastroenterology*, *35*(5), S79-S85.

49. Otsuki, T., John S, and Mirvat, S. Saving two in a billion: Quantifying the trade effect of European food safety standards on African exports. *Journal of Food Policy,26 (5)*

50. Pavlica, D. and Samuel, I. (1970). Primary carcinoma of the liver in Ethiopia. A study of 38 cases proved at post-mortem examination. *British Journal of Cancer, **24**(1)*, 22.

51. Pitt, JI Hocking, Ailsa D Glenn, Diane R (1983). An improved medium for the detection of Aspergillus flavus and A. parasiticus. *Journal of Applied Microbiology.*

52. Pitt, J.I., Hocking, A.D., Miscamble, B.F., Dharmaputra, O.S., Kuswanto, K.R., Rahayu,E.S. &Sardjono, T. (1998). The mycoflora of food commodities from Indonesia. *Journal of Food Mycology. 1:* 41–60.

53. Phillips, Timothy D Sarr, A Bashir Grant, Patrick G (1995). Selective chemisorption and detoxification of aflatoxins by phyllosilicate clay. *Journal of Natural Toxins, 3,(4).*

54. Ramesh V. B. and Siruguri V. (2003). Mycotoxin Food Safety Risk in Developing Countries. *Journal of International Food Policy Research Institute, Focus,10*

55. Rapid Alert System for For Food and Feed (RASFF).Retrieved from the Rapid Alert System for For Food and Feed website https://webgate.ec.europa.eu_*Referenec 2008.CAX*

56. Reddy,D Kiran Mayi,M Uma (2001). Aflatoxin B1 in different grades of chillies (Capsicum annum L.) in India as determined ny indirect competitieve-ELISA. *Journal of Food Additives & Contaminants.*

57. Rehima Mussema (2006). Analysis of red pepper marketing: The case of alaba and siltie in SNNPR of Ethiopia. Haramaya University.

58. Robards, Kevin Jackson, Peter E Haddad, Paul R (1994). *Principles and practice of modern chromatographic methods.* Academic Press London, UK.

59. Rosemary H. Waring, Glyn B. Steventon, Steve C Mitchell (2007). *Molecules of death.* Imperial College Press, second edition.

60. Scott P, Kennedy B, (1973). Survey of ground black, white and capsicum peppers for aflatoxins. *Journal of Association of Agricultural Chemists .56,* 1452–1457.

61. Sinha, K. K. (1998). *Detoxification of mycotoxins and food safety* (pp. 381-406). Marcel Dekker publication: New York.

62. Shankaran, R., Raj, H. G., & Venkitasubramanian, T. A. (1970). Effect of aflatoxin on carbohydrate metabolism in chick liver. *Enzymologia, 39*(6), 371-378.

63. Shephard, G.S. (2003). Aflatoxin and food safety: Recent African perspective. *Journal of Toxicology 22,* 267-286.

64. Shephard, G.(2008). Impact of mycotoxins on human health in developing countries. *Food Additives and contaminants, **25(2),*** 146-151.

65. Sobolev, V. S. (2007). Simple, rapid, and inexpensive cleanup method for quantitation of aflatoxins in important agricultural products by HPLC. *Journal of Agricultural and Food Chemistry, 55*(6), 2136-2141.

66. Stark, A. (1980). Mutagenicity and carcinogenicity of mycotoxins: DNA binding as a possible mode of action. *Annual Reviews in Microbiology, 34*(1), 235-262.

67. Sweeney, M.J. and Dobson, A.D.W. (1998). Mycotoxin production by Aspergillus, Fusarium and Penicillium species. *International Journal of Food Microbiology, 43,* 141-158.

68. Turner, Paul C Moore, Sophie E Hall, Andrew J Prentice, Andrew M Wild, Christopher P (2003). Modification of immune function through exposure to dietary aflatoxin in Gambian children. *Journal of Environmental Health Perspectives,111(2)*

69. USAID (2012). *Aflatoxin: A synthesis of the research in health, agriculture and trade.* Aflatoxin desk study Final report Retrived from the website http://www.aflatoxinpartnership.org.

70. Wagacha JM & Muthomi JW (2008). Mycotoxin problem in Africa: current status, implications to food safety and health and possible management strategies. *International Journal of Food Microbiology* ***124***,1-12

71. Wicklow, D.T., Hesseltine C.W., Shotwell, O. L. and Adams G.L. (1980). Interference competition and aflatoxin levels in corn. *Journal of Phytopathology, 70,* 761-764.

72. Williams, J.H., Phillips, T.D., Jolly, P.E., Stiles, J.K., Jolly, C.M. and Aggarwal, D. (2004). Human aflatoxicosis in developing countries: a review of toxicology, exposure, potential health conse-quences, and interventions. *American Journal of Clinical Nutrition 80,* 1106-1122.

73. Wilson Norbert (2007). *A review of empirical studies of the trade and economic effects of food-safety regulation.* Kluwer Academic Publishers.

74. Wogan, G.N. (1966). Chemical nature and biological effects of the aflatoxins. *Bacteriological Review* ***30,*** 460-470

75. Wu, Felicia (2004). Mycotoxin risk assessment for the purpose of setting international regulatory standards. *Environmental Science & Technology, 38 (5).*

76. Zinedine, A. and Mañes, J. (2009). Occurrence and legislation of mycotoxins in food and feed from Morocco. *Journal of Food Control, 20,*334-344.

Annex 1. Questionnaire for data collection-Farmers store

ADDIS ABABA UNIVERSITY

CENTER FOR FOOD SCIENCE AND NUTRITION PROGRAM

Identification:

Name of locality_____

Sample and Respondent code No_____

Verbal consent before conducting the interview

Instruction: My name is_____ I am a post graduate student at Addis Ababa University and conducting research, entitled: Occurrence of aflatoxins in red pepper (Capsicum *annum L.* Var."Mareko Fana"): In relation with storage and Grading. Your farm store has been selected to be included in this study. The finding of the study will be used as a basis for better planning of to prevent possible aflatoxin contamination. Therefore, I am requesting you to respond kindly to the questions and provide me access to your response. Your response will be kept confidential and all information collected from you will not be given to a third party. Your participation is voluntary and you are kindly requested to answer every question and allow me to observe the status of aflatoxin contamination in relation with conducive conditions for the contamination and will help to understand the post-harvest handling practices and possible contamination with aflatoxins. You will be beneficiary from the study. Are you willing to participate in this study?

1. If yes, please proceed to the next question;

2. If no pass to the next participant.

I. General

a. Are you aware of moldiness of pepper? [Code A1]

☐ Yes ☐ No

b. Do you use proper way of drying peppers? [Code A2]

☐ Yes ☐ No

By what mechanism you dry the pepper you produce?

☐ Sun drying intact with soil ground ☐ Drying machines

☐ Other (Please specify)_____

c. Do you use packaging suitable for aeration? [Code A3]

☐ Yes ☐ No

What type of packaging you use to wrap and store the pepper?

☐ Polyethylene bags ☐ Gunny bags

☐ Other materials (Please specify)_____

d. Do you give grades to the pepper you produce based on quality or other parameters before you sell your produces to merchants? [Code A4]

☐ Yes ☐ No

If your answer is yes please indicate your basis for grading?

---

e. Do you have a practice of pouring water to the pepper you produce before selling it to merchants? [Code A5]

☐ Yes ☐ No

If yes please indicate your reason for doing so?

II. Post-harvest handling (Drying conditions)

No	CRITERIA	YES	NO
1	Do you use clean and dry polyethylene sheets, cemented/concrete drying yards, drying beds or any other means to prevent contamination with soil. [Code B1]		
2	Do you frequently stir the red pepper while drying? [Code B2]		
3	Do you take a minimum of three weeks for drying the red-pepper after harvest? [Code B3]		
4	Do you discard peppers with problems before drying? [Code B4]		
5	Do you remove extraneous matters like plant parts before drying? [Code B5]		
6	Do you remove the stems from your pepper before drying? [Code B6]		
7	Do you have a mechanism for protecting the peppers from rain and pests while drying? [Code B7]		

III. Storing conditions

NO	CRITERIA	YES	NO
1	Do you use dunnage or kept 50-60cm away from the wall or floor to stack the packed bags while storing your pepper? [Code C1]		
2	Do you store your produce for more than 6 months? [Code C2]		
3	Does your store is well prevented from access by insects,rodents and other animals ? [Code C3]		

IV. Summary

Question Code	No. of respondents Rating YES	Rating (%)	Number of Respondents Rating NO	Rating (%)
A1	20	100	0	0
A2	18	90	2	10
A3	6	30	14	70
A4	0	0	20	100
A5	0	0	20	100
B1	2	10	18	90
B2	4	20	16	80
B3	4	20	16	80
B4	3	15	17	85
B5	3	15	17	85
B6	0	0	20	100
B7	5	25	15	75
C1	0	0	20	100
C2	3	15	17	75
C3	0	0	20	100

Annex 2. Questionnaire for data collection-Wholesalers store

ADDIS ABABA UNIVERSITY

CENTER FOR FOOD SCIENCE AND NUTRITION PROGRAM

Identification: _____

Sample and Respondent code No_____

Verbal consent before conducting the interview

Instruction: My name is_____ I am a post graduate student at Addis Ababa University and conducting research, entitled: Occurrence of aflatoxins in red pepper(Capsicum *annum L.* Var."Mareko Fana"): In relation with storage and Grading. Your store has been selected to be included in this study. The finding of the study will be used as a basis for better planning to prevent possible aflatoxin contamination in red pepper. Therefore, I am requesting you to respond kindly to the questions and provide me access to your response. Your response will be kept confidential and all information collected from you will not be given to a third party. Your participation is voluntary and you are kindly requested to answer every question and allow me to observe the status of aflatoxin contamination in relation with conducive conditions for the contamination and will help to understand the post-harvest handling practices and possible contamination with aflatoxins. You will be beneficiary from the study. Are you willing to participate in this study?

1. If yes, please proceed to the next question;

2. If no pass to the next participant.

I. General

a. Are you aware of moldiness of pepper? [Code D1]

☐ Yes ☐ No

b. Do you use packaging suitable for aeration? [Code D2]

☐ Yes ☐ No

What type of packaging you use to wrap and store the pepper?

☐ Polyethylene bags ☐ Gunny bags

☐ Other materials (Please specify)_____

c. Do you give grades to the pepper you produce based on quality or other parameters before you sell your produces to merchants? [Code D3]

☐ Yes ☐ No

If your answer is yes please indicate your basis for grading?

_____

d. Do you have a practice of pouring water to the pepper you produce before selling it to merchants? [Code D4]

☐ Yes ☐ No

If yes please indicate your reason for doing so?

II. Storing conditions

No	CRITERIA	YES	NO
1	Do you use dunnage or kept 50-60cm away from the wall or floor to stack the packed bags while storing your pepper? [Code E1]		
2	Do you store your produce for more than 6 months? [Code E2]		
3	Does your store is well prevented from access by insects,rodents and other animals ? [Code E3]		

III. Summary

Question Code	No. of respondents Rating YES	Rating (%)	No. of Respondents Rating NO	Rating (%)
D1	20	100	0	0
D2	3	15	17	75
D3	20	100	0	0
D4	20	100	0	0
E1	0	0	20	100
E2	15	75	5	25
E3	1	5	19	95

Submitted by

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Chairman Name _____

Signature _____ Date _____