ASSESSEMENT OF MYCOTOXIGENIC FUNGI AND THEIR TOXINS IN COFFEE (*Coffea arabica* L.) FROM MAJOR COFFEE GROWING AREAS OF JIMMA ZONE, SOUTHWEST ETHIOPIA

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NOVEMBER, 2019

JIMMA , ETHIOPIA

JIMMA UNIVERSITY

COLLEGE OF NATURAL SCIENCES

DEPARTEMENT OF BIOLOGY

SCHOOL OF GRADUATE STUDIES

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A Thesis Submitted to the Department of Biology, College of Natural Sciences, Jimma University, in partial fulfillment of the Requirement for the Degree of Master of Science in Biology (Applied Microbiology)

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November, 2019

Jimma, Ethiopia

DEDICATION

This Thesis work is dedicated to my beloved father Hagos Atsbeha, who had a great dream for my success in education but passed away without seeing those long journeys.

STATEMENT OF THE AUTHOR

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Name: Legese Hagos signature.....

Place: Jimma University, Jimma

Date of submission: November, 2019

ACKNOWLEDGMENTS

First and most, I would Praise the Almighty God that has given me the courage to start this Thesis and strength to finish it.

I would like to express my heartfelt gratitude to my academic supervisors Professor Ketema Bacha and Assistant Professor Meseret Guta, for their guidance, encouragement, visits to my study laboratory and professional expertise in bringing this work to completion. The valuable comments and constructive criticism they have given me throughout the period of the research work and write up of the Thesis were critical for my ultimate success. I really appreciate all the professional inputs and advices. I am grateful to Associate Professor Delelegn Woyessa and Mr. Leta Lejisa for evaluating the thesis work.

Ethiopian Agricultural Research Institute is duly acknowledged for giving me the opportunity to pursue graduate study by providing me study leave and financial support. The effort made by Jimma University College of Natural Sciences, Department of Biology in facilitating the courses and other materials is highly appreciated. Jimma Agricultural Research Center is highly appreciated for facilitating laboratory and vehicle throughout the research work. I would like to thank agricultural offices, coffee washers and farmers of Mana, Goma, Gera and Limu Kosa districts of Jimma zone for providing information and samples during the survey time.

My immense appreciation also goes to Jimma Agricultural Research staff, Demelash Teferi, Kifle Belachew, Gabisa Gidisa, Negasa Dechasa, Sisay Kidanu, Sisay Tesfaye and Ebrahim Ali for their lovely cooperation. I would like to express gratefulness to my lovely friends, Dr. Kumlachew Alemu, Dr. Binyam Tsedaley, Goytom Berihun, Tamene Hile, Mihrete Getnet, Yasin Oljira,Alemayehu Gudisa and Shihan Zerihun Asfaw for moral and technical support.

Finally, I would like to extend my thanks to my classmate students for their kindness and friendliness.

LIST OF ACRONYMS AND ABBREVIATIONS

CBB	Coffee berry borer
CYA	Czapek Yeast Extract Agar
CZA	CzapekDox Agar
CY20S	Czapek Yeast Extract Agar with 20% sucrose concentration
EC	European Commission
EU	European Union
FAO	Food and Agricultural Organization
HPLC	High Performance Liquid Chromatography
IARC	International Agency for Research on Cancer
LC	Liquid Chromatography
LSD	List Significant Difference
m.a.s.l	meters above sea level
MEA	Malt Extract Agar
OTA	Ochratoxin A
PBS	Phosphate Buffered Saline
RH	Relative Humidity
USDA	United State Department of Agriculture

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ABSTRACT

Coffee is the most important agricultural export commodity to fetch foreign currency. It needs to fulfill the quality standards of safety up to maximum tolerable level in order to get acceptance from international coffee markets. However, coffee is naturally associated with several mycoflora and some of them may produce Ochratoxin A unless careful handling measures are in place. Therefore, this research was initiated with the objectives of identifying mycotoxigenic fungi associated with coffee todetect and to quantify Ochratoxin A from coffee beans collected from the soil, wet and dry processing coffee mills. A total of 77 coffee samples were collected from Mana, Goma, Gera and Limu Kosa districts of Jimma zone, Oromia Regional State of Ethiopia. Malt Extract Agar (MEA) was used for isolation and identification of fungi associated with coffee using macro and microscopic characteristics. HPLC was used to detect and quantify Ochratoxin A from green coffee bean. Different fungal genera, including: Aspergillus, Fusarium, Penicilium and Rhizopus were found associated with coffee beans from different coffee processing methods having different altitudes. Accordingly, Aspergillus spp (84.74 %), Fusarium spp (8.75%), Penicillium spp (5.49%) and Rhizopus spp (1.02%) were recorded. The mean ochratoxin A (OTA) detected from different coffee samples were; 6.24µg/kg, 2.05µg/kg and 1.2µg/kg from soil, dry and wet processed samples, respectively. Mean ochratoxin A concentration level was the highest in coffee collected from soil (6.24µg/kg) while the quantity was relatively the least from wet processed coffee samples. Likewise, the highest fungal incidence was encountered in coffee beans collected from soil and the lowest fungal incidence was observed in wet processed coffee beans. The present study revealed that the degree of importance of mycotoxigenic fungi in Jimma zone. To manage the mycotoxigenic fungi and their mycotoxins, avoiding collecting coffee from soil, controlling insect pest infestations, avoiding mold formations, controlling moisture content of coffee are recommended to get high quality and market competitive coffee beans.

Keywords: *Aspergillus*, Coffee, HPLC, Jimma, Ochratoxin A

1. INTRODUCTION

1.1 Background and Justification

Coffee is one of the highly preferred international beverages and the most important traded commodities in the world next to petroleum (FAO, 2015). Coffee is the most important export commodity for Ethiopia and it accounts for about 34% of value of all exports (FAS, 2019). Ethiopia is the largest coffee producer in Africa &5th in the world next to Brazil, Vietnam, Colombia and Indonesia. It covers about 7.4% of total world coffee production (FAS, 2018). Sixteen percent of the Ethiopian population depends on coffee production, processing and marketing (ICO, 2016). Ethiopia exported around 180,000 metric tons of coffee and earned 800 million US$ (ICO, 2016). Ethiopia is known for its diverse and unique Arabica coffee flavors and the largest Africa's coffee producer and exporter (USDA, 2012). However, there are several constraints that remarkably minimize the profitability of Arabica coffee.

Mycotoxins are one of the most significant contributors to food and feed losses in developing countries. Fungal contamination and production of mycotoxins is one of the post-harvest problems that influence the quality of coffee beans. The storage fungi, mainly several species of *Aspergillus* and *Penicillium* do not invade seeds to any appreciable degree before harvest. However, they can cause severe discoloration of seeds in storage resulting in germination failure, due to damaged embryos or whole seeds (Malaker *et al*., 2008). Temperature, moisture content, storage conditions, processing type, and durations of storage can be important factors for mold development (Amezqueta *et al*., 2009). These mycotoxins have been implicated in acute mycotoxicosis in both humans and farm animals (Ramesh and Siruguri, 2003). Mycotoxins can develop during production, harvesting, or storage of grains, nuts, and other crops (Ramesh and Siruguri, 2003). Exposure of humans for long period of time to mycotoxin contaminated diets has been linked to kidney and liver cancer as well as weakening of the immune-system (Rios *et al*., 2013).

Ochratoxin A frequently occurs as a contaminant in various food products and it is the main mycotoxin reported to be found in coffee in green bean, roasted coffee and instant coffee worldwide (Taniwaki *et al.,* 2003; Almeida *et al*., 2007).OchratoxinA (OTA) shows

carcinogenic, nephrotoxic, teratogenic, immunotoxic, and neurotoxic properties. OTA is a small molecule soluble in water and it is chemically constituted by a combination of an amino acid (phenylalanine) and a polyketide to carbon 10, contains one chlorine atom necessary for its biological activity, and it is stable when exposed to heat (Bueno *et al.,* 2013).The presence of OTA in coffee is undesirable and its latent negative economic impact is usually enormous since it acts as a barrier to trade and potentially affects the economies of producing countries like Ethiopia.

The sensitivity of world community for food safety is increasing from time to time and this toxic metabolite still regularly receives attention and hits headline as potential health risks in human and animals. Many food processors in Europe and around the world are regularly testing for the presence of OTA in commodities like coffee before destined for human consumption (FAO/WHO, 2001). International Agency for Research on Cancer (IARC, 1993) classified OTA as group 2B (possible carcinogens to human). Due to its negative health impact in human, EU has set maximum permitted level of OTA 5µg/kg for roasted and ground coffee and 10µg/kg for soluble coffee (EC, 2006).

1.2 Statement of the problem

Ochratoxin A is a toxic fungal secondary metabolites occurring mainly in coffee and cereal grains. This mycotoxin is categorized as group IIB, possible carcinogen to human by IARC and it is the second most significant mycotoxin following Aflatoxins (IARC, 1993; Pitt, 2000). In Ethiopia, little is known about the mycofloral and potential ochratoxigenic fungal distribution in different agro-ecological zones and coffee processing systems. However, the occurrence of some ochratoxigenic fungi like *Aspergillus ochraceus* and other potential OTA producing genera of *Aspergillus, Penicillium* and *Fusarium* were encountered in coffee beans sampled from Jimma, Gera and Teppi areas, southwestern part of Ethiopia (Abraham, 2006; Eshetu and Girma, 2009). There was also scanty information available on the concentration of OTA in West Wollega, Ethiopia that indicated, 0(not detected) ppb, 1.24 ppb, 2.04 ppb OTA from Haru, Homa and Nedjo districts respectively (Legese *et al*., unpublished data). Geremew *et al*.,(2016) have reported that the mean OTA level of coffee in Ethiopia is 1.5µg/kg from different samples taken in some districts of Jimma zone using Enzyme Linked Immuno Sorbet Assay(ELISA) which is less sensitive than HPLC.

The current study fills the gap that ochratoxigenic fungi and the concentration of ochratoxin A in coffee beans from soil, wet and dry processing methods using the most sensitive detection method High Performance Liquid Chromatography (HPLC). Since OTA is a global issue, it needs much attention as it will have detrimental effect on the health of the society. Moreover, mycotoxin contamination may be a threat for Ethiopian economy as the country largely depends on coffee export earnings. OTA contaminations may result in rejection of coffee in the international market. The current study is initiated to assess the microbial distribution of Arabica coffee beans, potential mycotoxigenic fungi, and their ochratoxin content of dry and wet processing types from farmer's storage and coffee washers in Mana, Limu Kosa, Gomma and Gera districts of Jimma zone.

1.3 Objectives

1.3.1 General Objective

- To assess the occurrence of mycotoxigenic fungi associated with Arabica coffee and their mycotoxin levels among coffee beans collected from soil, wet and dry processed coffee beans in Jimma.

1.3.2 Specific Objectives

- ✓ To isolate and identify coffee bean associated fungi from Arabica coffee beans
- ✓ To characterize ochratoxigenic fungi from green Arabica coffee beans
- ✓ To assess the occurrence of mycotoxin in Arabica coffee beans
- ✓ To determine the concentration of Ochratoxin A in Arabica coffee beans using HPLC

1.4 Significance of the Study

Global coffee consumption continues to increase at an annual rate of 2.5-3%. About 125 million people in more than 60 developing countries depend on coffee for their food security and livelihoods (ICGN, 2017).

The current world market needs premium quality of the products and the producers have to fulfill the maximum tolerable quality requirements. One of the most deleterious effects in coffee quality is OTA contamination. EU has set maximum permitted level of OTA 5µg/kg for roasted and ground coffee and 10µg/kg for soluble coffee (EC, 2006). Results above the tolerable limits will cause total rejection. Therefore, the present study would provide the knowledge and understanding of microorganisms associated with coffee bean and their effect on the quality of coffee. It would also help to produce quality and to take immediate action when it exceeds beyond the maximum limit. It would reveal the current status of microbial distributions. In addition to this, it would assess the potential of mycotoxigenic fungi to cause mycotoxins in Arabica coffee beans to determine and correlate with maximum limit set by the EC. Finally, it would provide information on the status of OTA in Ethiopia for stakeholders; Producers, Researchers, exporters, and consumers.

2. LITERATURE REVIEW

2.1 Mycotoxins

Mycotoxins are poisonous (toxic) secondary metabolites produced by many filamentous fungi belonging to the phylum Ascomycota. Bennett (1987) suggested a definition of mycotoxins as "natural products produced by fungi that evoke a toxic response when introduced in low concentration to higher vertebrates and other animals by a natural route." Some mycotoxins can have additional effects such as phytotoxicity or antimicrobial activity. Generally, mycotoxins exclude substances such as those referred to by Bennett (1987) as "mushroom and yeast poisons". The major fungi causing frequent and problematic contamination of foods and feeds with mycotoxins are members of the fungal genera *Aspergillus, Fusarium, and Penicillium* (Bennett, 1987; Sweeney and Dobson, 1998; Marin *et al*., 2013).

These mycotoxins have been implicated in acute mycotoxicosis in both humans and farm animals (Ramesh and Siruguri, 2003). Mycotoxins have been characterized in 1960s with the discovery of aflatoxins (Feddern, *et al*., 2013). Mycotoxins can develop during production, harvesting, or storage of grains, nuts, and other crops (Ramesh and Siruguri, 2003). Exposure of humans for long period of time with mycotoxin contaminated diets has been linked to kidney and liver cancer as well as weakening of the immune-system (Rios *et al*., 2013). Mycotoxins occur more frequently under tropical climate where high temperature and humidity are prevailing. Some mycotoxins are produced before harvest (DON, ergot); some occur following harvest (fumonisin, ochratoxin); and a few predominantly occur during storage (aflatoxin) (Ramesh and Siruguri, 2003).

2.1.1 Ochratoxin A

Ochratoxinsare toxic chemical substances which are produced by certain fungi (*Aspergillus ochraceus and Penicillium verrucosum*). Structurally, they have a particularity of containing a chlorine atom. Naturally, they are found in many plant products, such as cereals, coffee, beans, cocoa, and nuts. They have been also detected in products made from cereals, wine, beer, and grape juice, as well as in animal products, such as pig kidneys (Cerain, 2007). Ochratoxin A (OTA) shows carcinogenic, nephrotoxic, teratogenic, immunotoxic, and

neurotoxic properties. It has been also associated with nephropathy in humans. OTA is a small molecule soluble in water and it is chemically constituted by a combination of an amino acid (phenylalanine) and a polyketide to carbon 10, contains one chlorine atom necessary for its biological activity, and it is stable1 when exposed to heat (Bueno *et al*., 2013).

2.1.1.1 Chemistry of Ochratoxins

Ochratoxins (Ochratoxin A: OTA, Ochratoxin B: OTB and Ochratoxin C: OTC) are toxic metabolites of different fungi; their structure consists of a dihydro-isocoumarin moiety linked with a phenylalanine through an amide bond (Figure 1). Furthermore, OTA and OTC contain a parachlorophenol part as well. OTA ($C_{20}H_{18}ClNO_6$; IUPAC name: N-{[(3R)-5-chloro-8-hydroxy-3-methyl-1-oxo-3, 4-dihydro-1H-isochromen-7-yl] carbonyl}-L phenylalanine; molecular weight: 403.8) is a white, odorless, heat stable, crystalline solid agent (melting point: 168–173^{O}C) with poor aqueous solubility (Pohland *et al*., 1992). OTA does not completely disappear during baking furthermore, OTA resists against three hours of high pressure steam sterilization at 121^{o}C (Vidal *et al*., 2015).

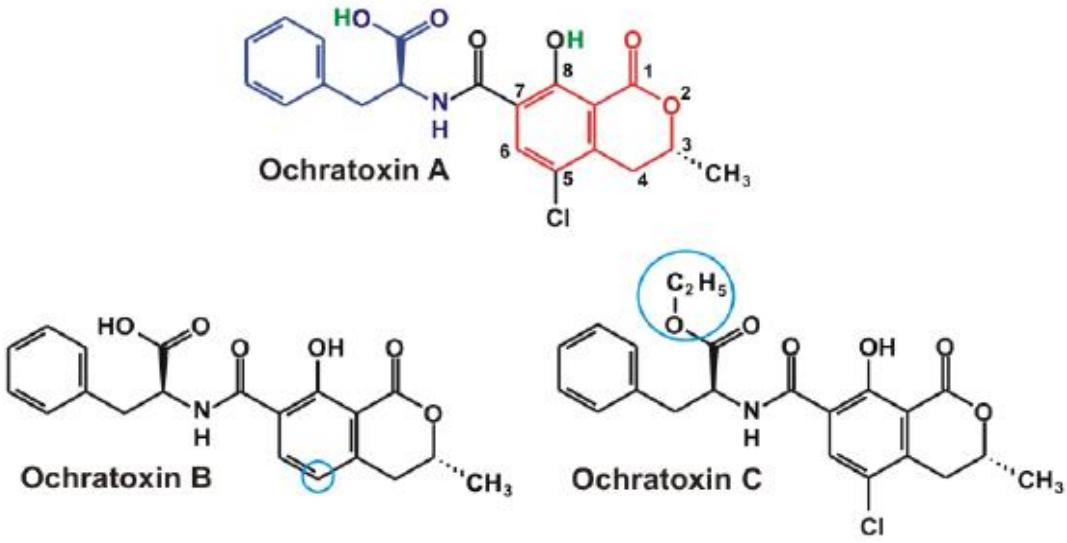


Figure 1.Chemical structures of Ochratoxin A, B, and C

(dark blue: phenylalanine part, red: dihydroisocoumarin ring, green: acidic hydrogens). The highlighted structures are characteristic to the three different ochratoxin molecules (light blue). Source; Kőszegi and Poór (2016).

Some countries have listed maximum tolerable limit for Ochratoxin A in coffee (table 1).

Table1. Countries list of maximum tolerable level for ochratoxin A in coffee (µg/kg).ICO (2005).

Country	Green coffee	Roasted coffee	Instant coffee
Czech Republic	10	10	10
Finland	5	5	5
Germany	-	3	6
Greece	20	-	-
Hungary	15	10	10
Italy	8	4	4
Netherlands	-	10	10
Portugal	8	4	4
Spain	8	4	4
Switzerland	5	5	5

2.1.1.2 OTA toxicity

OTA causes renal toxicity, nephropathy and immunosuppression in several species, and is carcinogenic in experimental animals. The nephrotoxic effects are related to the fact that OTA interacts with iron, forming a complex and producing hydroxyl radicals that promote lipoperoxidation. Renal damage is morphologically characterized by atrophy of the proximal tubule, broses and sclerosis. It is functionally characterized by incapacity of the tubular function, shown by a reduced ability to concentrate urine. No data are available on absorption, distribution, metabolism and excretion of OTA in humans. However, it has been reported that in vitro OTA binds with extremely high affinity to unidentified macromolecules in human plasma and to plasma proteins. The relative contribution of each excretory route for OTA varies with the species under study and the concentration of specific OTA metabolites in the urine (Pitt *et al*., 2000).

2.1.1.3 Human exposure to OTA

Dietary intake represents the main source of ochratoxin A in human. Human exposure to ochratoxin A occurs mainly through consumption of contaminated crops or intake of animal products, contaminated by carryover of OTA from contaminated feed (Scudamore *et al*., 1998). It is estimated that 12% of total OTA intake comes from coffee consumption worldwide (FAO, 1999). Since ochratoxin A was suggested to be a possible determinant of endemic nephropathy, considerable efforts have been made to determine a correlation between human exposure to this toxin and the incidence of the disease. Endemic nephropathy is a fatal human renal disease, recognized as a specific entity and affecting predominantly rural populations in limited areas of the central Balkan Peninsula. The disease has been reported in Bosnia and Herzegovina, Bulgaria, Croatia, Romania, and Yugoslavia (Serbia) (Taniwaki, *et al*., 1999).

2.1.2 Identification methods of ochratoxigenic filamentous molds

2.1.2.1 Cultural method

Cultural methods of identification for mycotoxigenic fungi that during mycelial differentiation certain cells enlarge, develop a heavy cell wall and form ‘T’ or ‘L’ shaped ‘foot cells’ (which are not separate cells) that produce a single conidiophore perpendicular to the long axis of the cell. Sometimes it is difficult to see the foot ‘cell,’ but when visible, morphologists take it as strong evidence that an isolate is an *Aspergillus* species. The erect hyphal branch developing from the foot cell is the conidiophore, which enlarges at its apex to form a rounded, elliptical or club shaped vesicle. The fertile area of the vesicle gives rise to a layer of cells called phialides (or steigmata in the older literature) that produce long chains of mitotic spores called conidia or conidiospores. The size and arrangement of the conidial heads as well as the color of the spores they bear are important identifying characteristics. For example, species in the *A. niger* group bear black spores, the *A. ochraceus* group is yellow to brown, while *A. fumigatus*, *A. nidulans*, and *A. flavus* are green. The major cultural features used in species identification are the color of the colony, the growth rate and thermo tolerance. *Aspergilli* have varying morphological and growth response to different nutrients so it is important to standardize conditions. Species identification depends upon pure cultures

grown on known media. The early taxonomic micrographs used a defined medium adapted from Czapek by Dox, often called 'Czapek Dox medium' which contains sucrose as the carbon source and nitrate as the nitrogen (Raper and Fennell, 1965).

2.1.2.2 Molecular method

Detection of OTA-producing *Aspergillus* using PCR methods is specific, sensitive, rapid, and easy to automate. (Atoui *et al*., 2007). Specifically, quantitative polymerase chain reaction (qPCR) is a powerful tool that combines fluorescent dyes and sequence-specific primers to monitor accumulation of PCR product during the procedure. Furthermore, it does not require other post-amplification procedures, such as gel electrophoresis. Thus, this technique is highly reliable, sensitive, and suitable for high throughput analysis (Mulè *et al*., 2006). Quantitative PCR has been used to quantify OTA-producing fungi in many agricultural products, including wine, cereal grain, tea, and coffee (Atoui *et al*., 2007). Common targets include polyketide synthase (*pks*), a gene involved in synthesis of secondary metabolites and OTA, as well as internal transcribed spacer (ITS) rRNA (Sartori *et al*., 2006; Atoui *et al*., 2007).

2.1.3 Factors governing mycotoxin production

Fungal growth and mycotoxin production are the consequence of an interaction among the fungus, the host and the environment. The appropriate combination of these factors determines the amount of colonization of the substrate, and the type and amount of mycotoxin produced. The synthesis of any particular mycotoxin depends not only on the species but also on the strain (Sweeney and Dobson, 1998). Many of the fungi capable of producing mycotoxins are also frequent contaminants of food and agricultural commodities. The mycotoxigenic fungi associated with the human food chain belong mainly to three genera: *Aspergillus*, *Penicillium* and *Fusarium*. While *Fusarium* species are destructive plant pathogens producing mycotoxins before or immediately after harvest, *Penicillium* and *Aspergillus* species are more commonly found as contaminants of commodities and foods during drying and subsequent storage. Mycotoxins cannot be produced unless fungal growth occurs. However, the presence of mycotoxigenic fungi in or on a food does not automatically mean the presence of mycotoxins, but that a potential for mycotoxin production exists. On the other hand, the absence of toxigenic fungi does not guarantee that the commodity is free of

mycotoxins, as the toxins may persist long after the fungi have lost their viability. The level of mycotoxin production is influenced by the nutrients available in the substrate. Environmental factors including temperature, water activity, gas atmospheres and preservatives also affect mycotoxin production (Pitt and Hocking, 1997; Sweeney and Dobson, 1998).

2.1.3.1 Water activity

Water activity is perhaps the most critical factor influences the germination, growth and establishment of molds on nutrient worthy substrates (Bouras *et al*., 2009). Esteban et al. (Esteban *et al*., 2006) were investigated the effect of different water activity values on OTA production using twelve *A.niger* isolates, cultured on Czapek Yeast Agar(CYA) and on Yeast Extract Sucrose agar (YES) where aw ranged from 0.82 to 0.99. For *A. niger*, it is known that minimal aw needed for fungal growth is 0.77 (Samson and van, 1988), but for OTA production it is found that optimal aw is 0.90-0.99, depending on the strain and culture

2.1.3.2 Temperature

After water activity, second limiting factor for OTA production is temperature. Optimal temperature for OTA production is between 25-30°C for *A. ochraceus* (Ramos *et al.,* 1998), 10-20°Cfor *A. carbonarius* (Bellí *et al*., 2005) and for *A. niger* "aggregate" 20-25°C (Esteban *et al*., 2004). Because of their ability to grow in a wide range of temperatures; OTA can be constantly formed in field. This fact is very important for some products, such as grapes and dried vine fruit, because main contaminants are the species of *A. carbonarius* and a few species from *A. niger* "aggregate", also main producers of OTA.

2.1.3.3 Gas tension

Moulds are obligate aerobes (require free oxygen for growth).Both reduction in oxygen tension and increase in carbon dioxide concentrations can have profound effects on the growth of fungi and production of toxin. These factors are important in the storage of commodities, where such conditions are often generated primarily for the control of insects (Moss, 1991). Paster *et al*., (1983) reported that OTA production by *A.ochraceus* was completely inhibited by >30% CO_2 on agar-based media after 14 days. This suggests that for efficient storage of moist cereals >50% CO_2 concentrations needs to be achieved rapidly to prevent OTA contamination in storage or during transport (Naresha, 2007).

2.1.4 Aflatoxins

The word aflatoxin is the combination of three words “a” for *Aspergillus* genus, “fla” for the species *flavus* and toxin, meaning poison (Feddern, *et al*., 2013). Aflatoxins are characterized by colorless to pale-yellow crystals, intensely fluorescent to ultraviolet light, emitting blue (aflatoxins B1 and B2) or green (aflatoxin G1) and green–blue (aflatoxin G2) fluorescence, from which the designations B and G were derived and aflatoxin is classified in to four common groups as B1, B2, G1, and G2. Contaminated grains and byproducts of grain are the most common sources of aflatoxin. Aflatoxin B1 (figure 2.) is recognized by the International Agency for Research on Cancer as one of the most naturally occurring toxic and carcinogenic substances found in nature (Feddern, *et al*., 2013). Aflatoxin producing fungi spp. and the moulds naturally originate in the soil and decayed vegetation in which risks of contamination begins with planting and can be worsened later during post-harvest practices through inappropriate harvesting, handling, storage, processing, and transport practices (Ephrem, 2015).

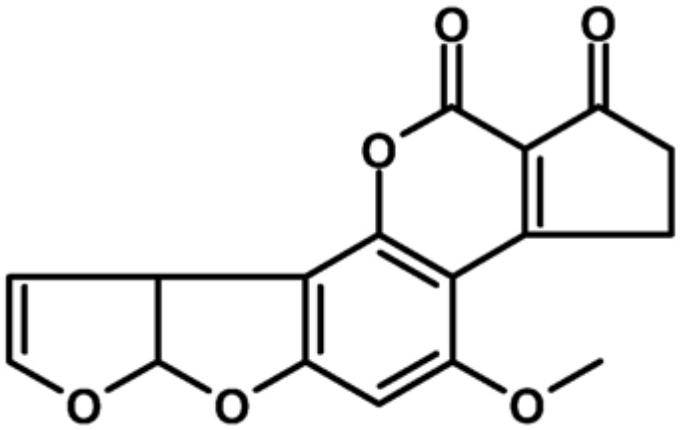


Figure 2. Chemical structure of aflatoxin B1 as a representative of the aflatoxins. Source; Richard (2007).

2.1.5 Fumonisns

Fumonisins have been extensively studied as mycotoxins produced by *Fusarium* species that have cancer-promoting activity (Gelderblom *et al*., 1991) as well as being associated with a number of animal and human diseases (Harrison *et al*., 1990; Marasas, 2001). The fumonisin B (FB) molecule is composed of a long hydroxylated hydrocarbon chain with added

tricarballylic acid and methyl and amino groups (Gelderblom *et al*., 1991). FB1, FB2, and FB3 are the major naturally occurring FBs. FB1 is by far the most prevalent of the fumonisins in the human diet and is categorized as a Group 2B carcinogen by the International Agency for Research on Cancer (IARC, 2002). Fumonisins, which inhibit the uptake of folic acid via the folate receptor (Stevens and Tang, 1997), have also been implicated in the high incidence of neural tube defects in rural populations known to consume contaminated maize, such as in the former Transkei region of South Africa and some areas of Northern China (Marasas *et al*., 2004). Frisvad *et al*., (2007) reported that a number of industrially important strains of *Aspergillus niger also* produce FB2 and FB4, additionally pointing out the potential mycotoxicological risk of some foods colonized by this species. Mogensen *et al*., (2010) demonstrated that *Aspergillus niger* strains isolated from raisins are capable of producing both FB2 and FB4 when cultured on either grapes or raisins.

2.1.6 Zeralenon

This mycotoxin was mentioned earlier in that it may co-exist with DON as the same organisms, F. *graminearum* or F. *culmorum* (CAST, 2003), may produce both compounds. Chemically, it is a phenolic resorcyclic acid lactone that is estrogenic when consumed by animals, primarily swine (Hidy *et al*., 1977). Grains infected with the above organism may exhibit the pink color associated with the production of a pink pigment simultaneously produced with the zearalenone. Most often this mycotoxin is found in corn. However, it is found also in other important crops such as wheat, barley, sorghum and rye throughout various countries of the world (CAST, 2003). In wheat the conditions for the occurrence of zearalenone would be essentially the same as for the occurrence of DON since the organism gains entry into the host plant in the same manner. Generally, the *Fusarium* species grow in moist cool conditions and similarly invade crops under these more favorable conditions. In wheat, sorghum and corn, zearaleone occurs in pre-harvest grain (WHO Food Additives Series: 44, 2000).

2.1.7 Ergot Alkaloids

The ergot alkaloids are among the most fascinating of fungal metabolites. They are classified as indole alkaloids and are derived from a tetracyclic ergoline ring system. Lysergic acid, a

structure common to all ergot alkaloids, was first isolated in 1934. The clavines have ergoline as a basic structure but lack peptide components; the lysergic acid alkaloids include ergotamine and lysergic acid amide (ergine) (Bennett& Bentley, 1999). The human disease acquired by eating cereals infected with ergot sclerotia, usually in the form of bread made from contaminated flour, is called ergotism or St. Anthony's fire. Two forms of ergotism are usually recognized, gangrenous and convulsive. The gangrenous form affects the blood supply to the extremities, while convulsive ergotism affects the central nervous system (Bennett& Bentley, 1999).

2.1.8 Patulin

Patulin, is produced by many different molds but was first isolated as an antimicrobial active principle during the 1940s from *Penicillium patulum* (later called *Penicillium urticae* , now *Penicillium griseofulvum*) (Birkinshaw *et al*., 1943). The same metabolite was also isolated from other species and given the names clavacin, claviformin, expansin, mycoin c, and penicidin (Ciegler *et al*., 1971). A number of early studies were directed towards harnessing its antibiotic activity. Patulin is a metabolite produced by a large number of microscopic filamentous fungi within several genera such as *Bysochlamys*, *Eupenicillium* , *Penicillium* , *Aspergillus* and *Peacylomyces* in a variety of food products, e.g. apricots, grapes, grape fruit, peaches, pears, apples, olives and cereals (Kadakal and Nas, 2002). Nowadays, *Penicillium expansum* , the blue mold that causes soft rot of apples, pears, cherries, and other fruits, is recognized as one of the most common offenders in patulin contamination. Several studies have shown that patulin is stable in dry cereals, and in apple and grape juice, but that it is decomposed in wet cereals and during production of cider (Moss and Long, 2002). Residues of patulin can cause particular safety issues in products such as juices derived from apples and citrus fruits (Beretta *et al*., 2000).

2.2 Major Methods for detecting mycotoxins

2.2.1 Thin Layer Chromatography (TLC)

TLC was the first chromatographic method to be applied to mycotoxin determination and is still in routine use in many laboratories, especially in developing countries, for aflatoxin analysis. After suitable extraction and clean up, mycotoxins are readily separated on silica gel

TLC plates using any one of a number of solvent mixtures such as acetone–chloroform (1+9), benzene–methanol–acetic acid (90+5+5), ether–methanol–water (96+3+1) or chloroform–isopropanol (99+1) (Trucksess, 2001).

In thin layer chromatography (TLC), the stationary phase is polar and mobile phase is non-polar, allowing separation of the mycotoxin to be analyzed. The layer of adsorbent is known as the stationary phase. After the sample has been applied on the plate, a solvent or solvent mixture (mobile phase) is drawn up the due to capillarity. Because different analytes ascend the TLC plate at different rates, separation is achieved. TLC can be used to monitor the progress of a reaction, to identify compounds present in a given mixture, and to determine the purity of a substance. Specific examples of these applications include: analyzing ceramides, fatty acids, detection of pesticides, assaying the radiochemical purity of radiopharmaceuticals, and identification of components in medical plants (Reich, 2007).

2.2.2 Enzyme Linked ImmunoSorbet Assay (ELISA)

An ELISA method for mycotoxin assay is based on the ability of a specific antibody to distinguish the three-dimensional structure of a specific mycotoxin. The direct competitive ELISA is commonly used in mycotoxin analysis (Chu, 1996). A conventional microtiter plate ELISA requires equilibrium of the antibody–antigen reaction that would require an incubation time of approximately 1–2 h. currently, most of commercially available ELISA test kits for mycotoxins are working in the kinetics phase of antibody–antigen binding, which reduces the incubation time to minutes. Although reduction of incubation time may lead to some loss of assay sensitivity, the test kit can provide accurate and reproducible results.

After a mycotoxin is extracted from a ground sample with solvent, a portion of the sample extract and a conjugate of an enzyme coupled mycotoxin are mixed and then added to the antibody-coated microtiter wells. Any mycotoxin in the sample extract or control standards is allowed to compete with the enzyme-conjugated mycotoxin for the antibody binding sites. After washing, an enzyme substrate is added and blue color develops. The intensity of the color is inversely proportional to the concentration of mycotoxin in the sample or standard. A solution is then added to stop the enzyme reaction. The intensity of the solution color in the microtiter wells is measured optically using an ELISA reader with an absorbance filter of 450

nm. The optical densities (OD) of the samples are compared to the ODs of the standards and an interpretative result is determined (Trucksess, 2001).

ELISA test kits are favored as high throughput assays with low sample volume requirements and often less sample extract clean-up procedures compared to conventional methods such as TLC. The methods can be fully quantitative. They are rapid, simple, specific, sensitive and portable for use in the field for the detection of mycotoxins in foods and feeds (Trucksess, 2001).

2.2.3 High Performance Liquid Chromatography (HPLC)

The most common method used currently for mycotoxin 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 mycotoxins. 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 (Legesse, 2010).

2.3 Coffee growing

For coffee production, weather temperature is a key factor, and latitude and elevation strongly influence the temperature. Coffee is grown over a wide range of elevations at any given latitude. Coffee is grown around the world from 24°N to 25°S latitudes and ranging in elevation from sea level to as high as 2133 meter. Humid and hot environments can allow coffee trees to flourish. In countries at or near the equator, such as Kenya, the New Guinea highlands, and Colombia generally, high elevation coffee regions are found. Coffee growing

regions can occur in low-elevation subtropical latitudes (22–25°) such as Hawai'i and Sao Paulo, Brazil. (Bittenbender and Smith, 2008).

In the tropical forests of Ethiopia, Kenya, and Sudan, at altitudes of 1500 - 2800m, and between the latitudes of 4°N and 9°N, arabica coffee is the native species. In this region, the mean annual air temperature is between 18 and 22°C and shows little seasonal fluctuation (Camargo, 2010).

There are two main coffee species that are employed worldwide, arabica coffee and robusta coffee. Arabica coffee (*Coffea arabica* L.) accounts for approximately 70%of commercial production, and the remaining 30% is from robusta coffee (*Coffeacanephora* var. robusta). In Central and South America and in East Africa two-thirds of the coffee stems from *Coffea arabica* (van der Stegen, 2003). Arabica coffee originated from Ethiopia as it is indigenous to Africa, and the origin of robusta coffee is from the Atlantic Coast and the Great Lakes region in Africa (ITC, 2012).

2.4 Effect of coffee processing on prevalence of mycotoxins

2.4.1 Dry processing

This method involves the drying of red cherries without using water at any stage. The harvested ripe cherries spread over a raised bed, concrete, or on any other suitable drying material and raked at regular intervals to prevent fermentation and to ensure even drying. The cherries dried from moisture content of about 65% to 12%. The cherries are dried on beds constructed from chicken wire and fixed on wooden frames raised about 80 cm above the ground. Bucheli and Taniwaki (2002) noted that, in the cherry fruits, the skin particles generated after drying and processing containing OTA may be mixed with the healthy beans, compromising the final quality of the coffee. According to Bars and Bars (2000), the ochratoxigenic species of *Aspergillus* such as *A. ochraceus* are neither phytopathogenic nor endophytes; thus, contamination by OTA in healthy fruits would not be possible.

2.4.2 Wet processing

The wet method involves use of water in most stages. This method involves several stages whereby ripe cherries are transformed into parchment coffee. In full- fledged conventional

wet processing, red cherries are pulped, fermented under water until the mucilage is degraded so that it can be easily washed off. Fermentation period in most cases varies from 12 to 48 hours depending on the temperature of the locality. The parchment is then washed and dried to attain a moisture content of 10-11.5%. Regarding the samples processed by wet method, the results showed that the removal of coffee husk (cherry dehusking) or of the husk and mucilage (cherry despulping) reduced bean contamination, thus indicating that fungi and OTA contamination would be concentrated in the skin until before processing (Batista *et al*., 2009).

Frank (1999) reported that pulping would significantly reduce the risk of OTA contamination during subsequent fermentation and drying. According to Bucheli *et al*. (2000), coffee bean skin is the main substrate for the development of ochratoxigenic fungi. Besides eliminating a number of OTA producing microorganisms, skin removal accelerates drying, decreasing the risk of fungi development and OTA production. However, the initial quality of the fruits harvested the presence of OTA-producing fungi as well as the processing site conditions can certainly contribute to the formation of OTA in coffee processing by wet method (Bucheli and Taniwaki, 2002).

2.5 Effect of insect infestation in mycotoxins

Coffee Berry Borrer is a common insect pest in coffee plantations. This insect (*Hypothenemus hampei*, (Ferrari)), damages coffee berries, reduces coffee yield, and is a suspected vector of various mycotoxin producing molds (Velmourougane *et al*., 2010). It is useful to understand the role of CBB in the coffee growing regions of the world, especially in plantations for production of quality coffee. Throughout the world, CBB is a serious coffee pest, that has now spread to most coffee growing regions in Central Africa, and has been reported in Uganda, Congo, Benin, Togo, Ivory Coast, Kenya, Nigeria, Angola, Ethiopia, Brazil, Colombia, Guatemala, Ecuador, Nicaragua, Honduras, Mexico, Malaysia, Indonesia, Sri Lanka, Jamaica, New Caledonia, India, and other countries (Vega and Mercadier, 1998). The following fungal species have been isolated from coffee berry borers: *Aspergillus ochraceus, Aspergillus flavus, Aspergillus niger, Fusarium sp., Penicilliumchrysogenum, Penicillium*

brevicompactum and Verticillium spp. The isolation of *A.ochraceus* suggests that CBB might serve as a vector for this toxigenic fungus (Vega and Mercadier, 1998).

2.6 Economic importance of mycotoxins

The Food and Agricultural Organization has reported that 25% of the world's food crops are contaminated with mycotoxins. This is not only causes economic loss but also reduces the world's food supply. The contaminating fungi and mycotoxins are found in food crops as well as in a number of processed foods intended for human consumption. Mycotoxins pose higher risk of causing cancer than contaminants in food such as anthropogenic contaminants, pesticides, phycotoxins and food additives (Kuiper-Goodman, 1998).

Being responsible for large financial losses in conjunction with contaminated and thus unsafe food, beverages and feed as well as being linked to the genesis of several disease states, mycotoxins have been recognized as potential threat to both human and animal health and are under surveillance of international agencies attempting to define safe legal limits for concentrations in foodstuffs, processed food and in animal fodder (Shane, 1994).

There have been several outbreaks of mycotoxicosis in the human population. In 1974, an outbreak of hepatitis in India resulted in the death of 100 people due to consumption of contaminated maize (Krishnamachari *et al*, 1975). In another case in India, an outbreak of gastrointestinal disorder associated with consumption of bread made of contaminated wheat was reported in 1987. The Kenyan outbreak in 2004 was one of the largest outbreaks, where 125 people died due to liver failure caused by acute aflatoxicosis after consumption of contaminated maize (Muture and Oqana, 2005).

2.7 Management of mycotoxins

The growth of the mould and subsequent production of OTA is dependent upon several factors including temperature, humidity, and water activity during the harvesting, drying and storage of the crops (Dongo *et al.,* 2007). Ochratoxin contamination takes place from field to fork level hence there is a need to control OTA contamination at each level. To address this

problem there should be good agricultural practices, storage practices, strong regulator measures in the form of HACCP protocols, regulations and code of practice are needed.

Control at cultivation level: By Crop rotation, Selection of Resistant varieties and Avoid temperature and drought stress during the Seedling growth **Pre harvest level:** Minimize the insect and mechanical damage; Control of weeds, Harvest should be done on full maturity. **Harvest level:** Hygienic and handling practices, proper drying and cleaning are required without the other matter. **Storage practices:** Preservation of properly dried materials, Storage is done in well ventilated structures; Good housekeeping Home keeping procedures should be followed, Transportation also done in good condition

3. MATERIALS AND METHODS

3.1 Description of the Study Area

Jimma Zone is one of coffee growing zones in the Oromia Regional State, which has a total area of 1,093,268 hectares of land. Jimma town is the capital and administrative center of the zone, 335kms distance to Southwest of Addis Ababa, the capital city of Ethiopia. The total area of land covered by coffee in the zone is about 105,140 hectares, which include small-scale farmers' holdings as well as both state and private owned plantations. Out of the 40–55 thousand tons of coffee annually produced in the Zone (JZARDO, 2008), about 28-35 thousand tons is sent to the central market, while the remaining is locally consumed (Alemayehu *et al*., 2008). The Zone covers a total of 21% of the export share of the country and 43% of the export share of the Oromia Region (JZARDO, 2008). The zone is classified into three agro-climatic zones: Kolla (14.9% - lowlands); Woinadega (64.4% -mid Highlands); Dega (20.5% - highlands). Forest, semi-forest, garden and plantation coffee production system are exercised in the zone.

The study was conducted both in the field and laboratory. Field study was conducted in Mana, Limu kosa, Gomma and Gera districts of Jimma zone (Fig 3). The districts are located in south western part of Ethiopia. Mana is located in 7^{o}54'N36^{o}53'E,and its altitude is 2010m above sea level with annual average rainfall 1647mm and, Limu kosa is located in7^{o}56'N36^{o}38'E and its altitude is 1950m above sea level with annual average rainfall 1604mm and. Gera is located in 7^{o}70'N36^{o}00'E and its altitude is 1940m above sea level with annual average rainfall of 1645.3mm and relative humidity is 75.03% and. Gomma district is located in 7^{o}85'N36^{o}60'E and its altitude is 1666m above sea level with annual average rainfall of 1521.01mm and relative humidity 72.73% and. (Ethiopian meteorology agency, Jimma branch 11 years average of 2007-2017). The laboratory activities were conducted at pathological Laboratory of Jimma Agricultural Research Center and Bless Agri Foods Laboratory PLC. Legetafo, Ethiopia.

Table 2. Processing methods, Moisture content, Temperature, Humidity and Altitude of study areas.

Dry Processed coffee						
Districts assessed	kebele	No. of household	Moisture %	Temperature (^{O}C)	Humidity (%)	Altitude (m.a.s.l.)
Mana	Haro	5	15-18.4	26-29.6	31-50	1662-1675
	Kelaguda	5	14-17.5	26.3-30.1	37-48	1656-1726
	Botore	5	15.4-19	25-28.8	31-44	2105-2111
Gomma	Koysecha	5	13.3-15.1	29.1-32.8	23-31	1640-1765
	Omogurude	5	15-15.6	25.1-30.3	40-46	1697-1742
	Bulbula	5	15.2-17	25-29.5	42-43	1619-1639
Gera	Sediloyo	5	15-15.8	31.5-32.7	38-40	1944-1961
	Yukro	5	15.1-17.6	27.6-29.1	41-45	1946-1951
	Ginjichala	5	14.9-16.1	22.5-27	41-49	1976-2051
Limu Kosa	Suntu	5	12.8-16.2	24.6-29.4	51-62	1695-1711
	Debelo	5	13.9-15.4	25.9-29.9	45-46	1732-1738
	Mendera		13.2-15.3	24.9-32.4	42-45	1672-1685
Total	12	60				
Wet Processed coffee						
Mana	Mana	4	10.5-13.4	27-31	30-37	1600-1672
Gomma	Gomma	4	10-11.3	29.4-31.4	28-47	1640-2145
Gera	Gera	4	11.4-13	21-29.4	46-59	1928-1935
Limu Kosa	Limu Kosa	5	8.9-12.5	23.9-29.2	47-51	1700-1705
Total	4 Districts	17				

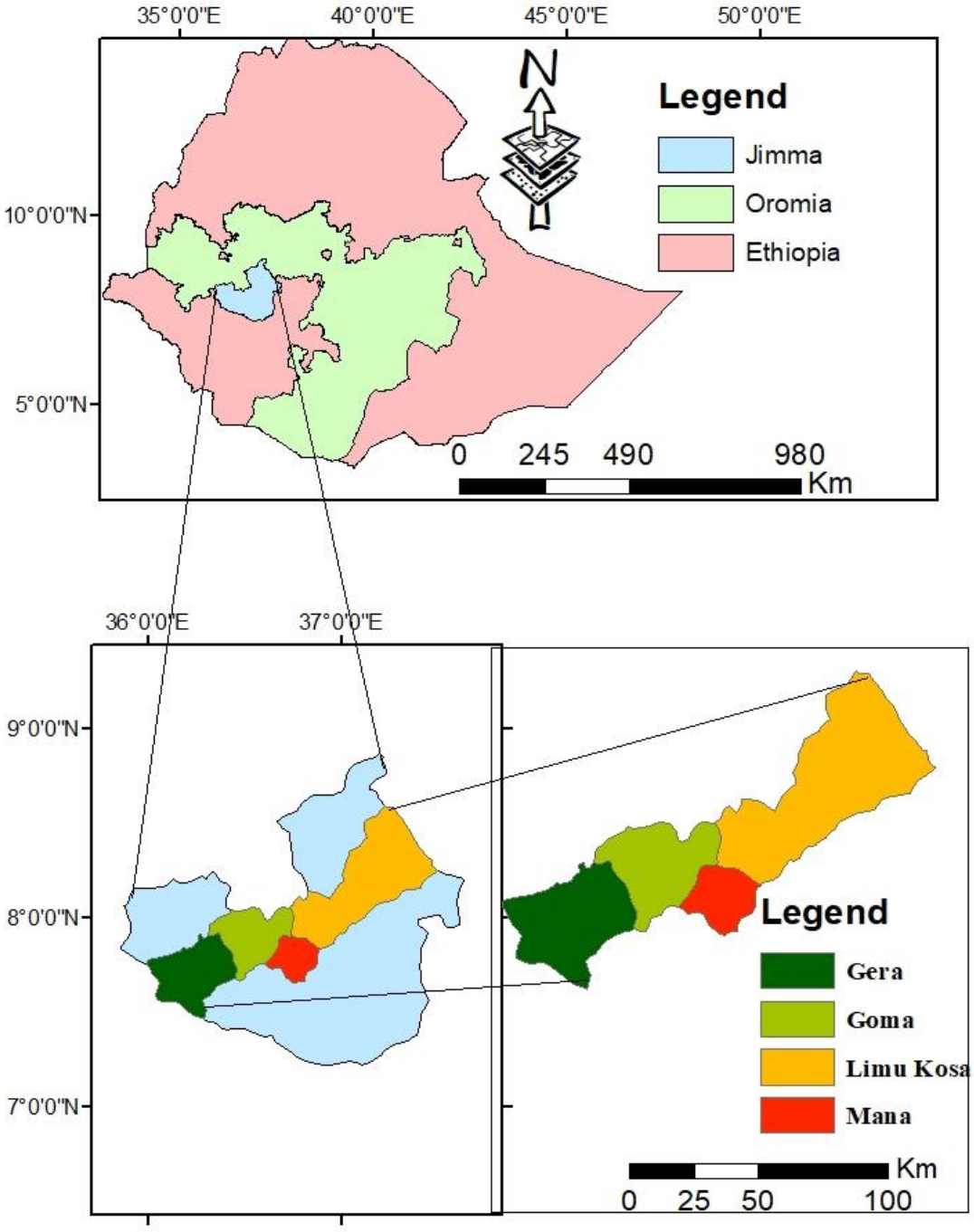


Figure 3.Map of sampling sites

3.2 Study Design, population, Sampling techniques and Sample collection

A cross-sectional study and laboratory experiment was conducted from February 2019 to July 2019. Multistage sampling procedures were employed to select coffee producing households. At first stage four sample districts (Mana, Gomma, Gera and Limmu Kosa) from Jimma were selected purposively. Three peasant associations per district and five coffee producer farmers per peasant associations were selected for dry processed coffee. In addition, four coffee washers per districts except Limmu Kosa where five washers were randomly selected. In the present study a total of 60 coffee producing farmers and 17 coffee washers were visited. Random sampling technique was conducted to collect samples from coffee producing farmers and coffee washers from each sampling site of the study areas.

From each district, three major coffee growing kebeles were selected based on the information gained from the agricultural office of the districts. From each kebeles, 5 coffee samples each weighing one Kg was randomly collected using perforated sterile polyethylene bag. This represents dry processed coffee type from each kebeles farmer's storage. A total of 17 samples were collected from coffee washers of the districts using perforated sterile polyethylene bag that represents wet processed coffee type. The storage time of the coffee samples was 4 months. Questionnaire was used to collect the necessary data associated with the storage conditions and related parameters. Accordingly, GPS reading, temperature, moisture content, storage duration storage method and pest condition were collected together with samples. The samples were taken to Jimma Agricultural Research Pathology Laboratory for isolation and identification of coffee bean associated fungi.

3.3 Temperature and relative humidity determination

Temperature and relative humidity of the storage condition in the respective study area was recorded by inserting to the storage material using thermo Hygro, Yueqing Xinyang Automation Equipment, Ltd Co., China.

3.4 Moisture content determination

Moisture content of each sample (cherry) and Parchment was recorded on sampling sites in a storage using digital moisture tester (Gehaka G600, Italy).

3.5 Visible mold over growth

One hundred coffee cherries were randomly selected from the samples and all the cherries were examined for the over growth of visible mold on the cherries and computed to percentage.

3.6 Coffee berry borer infestation

One hundred coffee cherries were randomly selected from the samples and all the cherries were examined for the presence of an entry hole (perforation) on the cherries, which is typical symptom of coffee berry borer attack (Le Pelley, 1968; Baker, 1999)

3.7 Isolation and identification of fungi

Isolation of mycobiota associated with coffee beans were conducted following the procedure employed by Samson *et al*., (2004). The coffee bean samples were surface sterilized with 5% sodium hypochlorite and was rinsed 3 times using sterilized distilled water. Then, 10 coffee beans were placed into a triplicate sterilized Malt Extract Agar Media amended with 1g streptomycin per1000ml of medium. The plates were incubated at 25°C for up to 7 days and the number of samples showing fungal growth in each Petri dish were counted and their frequency was computed using the following formula;

$$\text{Percent of Fungal infection (\%)} = \frac{\text{number of beans from which fungus isolated}}{\text{Total number of beans analyzed}} * 100$$

The growing fungi was sub cultured on Malt Extract Agar (MEA), CzapekDox Agar (CZA), Czapek Yeast Ager (CYA), and Czapek Yeast Sucrose Agar (CY20%S) for 7 days and identified to species level based on cultural and morphological characteristics, including colony color, conidiophores, phialids, presence and size of vesicles, surface texture, color and the reverse side of mould on agar plate, mycelium, type of spore, pigmentation, Vesicle, Hyphae, Metulaes, Arrangement of conidia (Raper and Fennell, 1965; Klich, 2002;

McClenny, 2005; Pandit *et al*, 2014). The pure cultures of each isolate were stored at 4^{o}C on MEA slant during the study.

3.7.1 Colony (mycelial) radial growth

Cultures of mycotoxigenic fungi were inoculated on MEA. Hyphal tip of each isolate was placed at the center of 20 ml MEA dispensed in a 96 mm diameter sterilized petridish with three replications. Mycelial (colony) radial growth (mm) of each isolate was measured with ruler, colony diameter from two perpendicular planes on the reverse side of the Petri-dishes.

3.7.2 Conidial Size, vesicle size and stipe diameter

Mycotoxigenic *isolates* were incubated on MEA medium for 7 days, replicated 3 times per isolate. Length and width of conidia, vesicle and stipe were measured with ocular micrometer (μm), which was fitted into 10x eyepiece and adjusted at 40x objective of the compound microscope.

3.8 Ochratoxin A determination in coffee bean by HPLC

Detection and quantification of ochratoxin A was conducted at Bless Agri Food Laboratory service PLC. Legatafo, Ethiopia. The Ochratoxin A detection and quantification from green coffee bean was conducted following the procedure employed by (Vargas *et al*., 2005).Based on total samples proportion from which 51 from dry processed, 9 samples from ground soil collected and 17 from wash processed coffee samples, 20 samples from all were selected for ochratoxin A detection and quantification. A total of 20 coffee samples (coffee collected from soil=3 from dry processed =13 and from wet processed =4 samples) were subjected to HPLC for detection and quantification of ochratoxin A from green coffee beans.

3.8.1 Sample preparation

A500g of green coffee was homogenized and finely grinded with green coffee bean grinding machine (FW 100, Huanghua Faithful Instrument Co. Ltd, China) to pass through 0.5-1.0 mm (80% of the material has been <0.5mm particle size) mesh size. After grinding, the grinder was cleaned with clean brush to prevent Ochratoxin A cross contamination. All the

batches of test materials, before and after packaging were stored at -18^{o}C and protected from light.

3.8.2 Extraction Procedure

Twenty five gram sample portion of grinded green coffee was weighed and added in to 500ml conical flask. Then, 200 ml of 3% aqueous sodium bicarbonate solution and the grinded green coffee (1+1) were blended for 5 minutes with a blender at speed of 8000rpm. Then after, the mixture was filtered through a folded Whatman qualitative filter paper with pore size of11 µm. After the filtration, the filtrate was collected and re-filtered through a fiber glass membrane with 2 µm pore size using vacuum system. After the second filtration, 4ml aliquot of filtered extract has been transferred to graduated cylinder then diluted to 100ml with Phosphate buffer saline and homogenized (Vargas *et al*., 2005).

3.8.3 Cleanup with immunoaffinity (OTACLEAN^{TM})

The immunoaffinity column has been connected to the vacuum manifold and then 60ml reservoir was attached. The immunoaffinity column was opened for use and the storage buffer was drained until the level reaches the upper fit. Diluted extract of 100 ml was taken and passed through OTACLEAN^{TM} column. All the samples were drained through the column until there was no more sample in the column (Vargas *et al*., 2005).

3.8.4 Elution of OTA

Four ml HPLC grade methanol was applied to the OTACLEAN and kept a side for 3 minutes to allow the solvent to permeate the gel before elusion. The ochratoxin was eluted from the column into 100 ml Beaker at a flow rate of 2-3 ml/min using positive pressure. The elute was evaporated to dryness using gentle stream of nitrogen in a block heater at 45^{o}C then the residue was re-dissolved with 1ml Liquid Chromatography mobile phase and homogenized.

3.8.5 Detection and quantification of OTA by HPLC

The mobile solvents were Acetonitrile: Methanol: Water (5:55:40) with flow rate of 1.2 ml/min and the column was stainless steel, reversed phase (C18) column, 250x4.6mm, with 5µm particles and the injection volume was 20 µm. HPLC (Shimadzu LC-10ADVP, Japan,

with a fluorescence detector) employed with excitation wavelength of 333 nm, emission wavelength of 460nm.The prepared standard ochratoxin solution (sigma-aldrich, USA) was injected. Finally the sample was injected.

3.9 Data analysis

Data on coffee bean associated fungi and level of ochratoxins were analyzed using descriptive statistics.

Means separation was performed with Tukey's Test. The associations of Ochratoxin A with independent variables *viz.* altitude, Temperature, humidity, moisture content, visible mold overgrowth, coffee berry borer damage, *A.ochraceus*, *A.niger*, *A.flavus*, *A.melleus* were analyzed using Pearson correlation and regression using SPSS 20.0 software package (Green and Salkind, 2016).

4. RESULTS

4.1 Total mycoflora associated with coffee bean

A total of four fungal genera, including *Aspergillus, Fusarium, Penicilium and Rhizopus* were found to be associated with coffee beans collected from different coffee processing facilities (wet and dry) and agro ecologies. From a total of 77 samples analyzed for coffee bean associated fungal genera, *Aspergillus* spp were the most prevalent (84.74 %), followed by *Fusarium* spp (8.75%), *Penicillium* spp (5.49%) and *Rhizopus spp* (1.02%) (Figure 4).

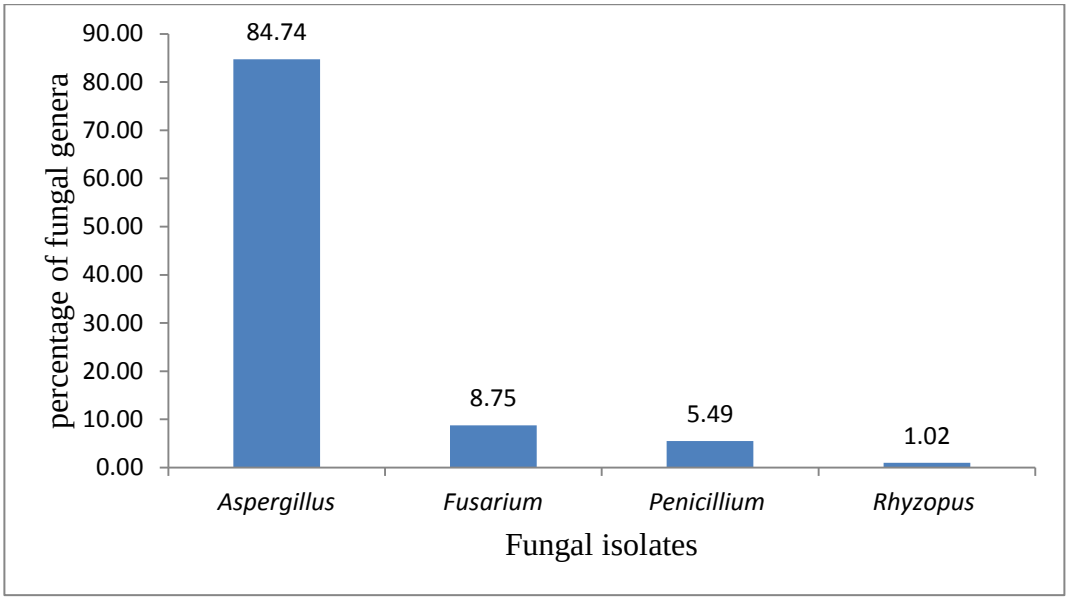


Figure 4.Frequency of isolation of major fungal flora associated with coffee bean

4.2 Fungi associated with different coffee processing type and from different coffee sampling method

The result of this study indicated that there was a difference in fungal population with in different coffee processing methods. The mycoflora that frequently found in association with coffee beans collected from soil, dry and wet processed samples were *Aspergillus* spp (76.3%, 63.4%, 28.8%), *Fusarium* spp (8.9%, 6.5%, 3.5%) *Penicillium* spp (7%, 3.6%, 0.4%), and *Rhizopus* spp (1.5%, 0.7%, 0.4%), respectively (Fig 5). Accordingly, *Aspergillus* spp dominated fungal isolates detected in coffee beans collected from different coffee processing facilities and soil, with coffee sampled from soil being the most contaminated beans.

Comparatively, wet processed coffee bean samples were less contaminated with *Aspergillus* spp although frequency of isolation of *Aspergillus* spp is still higher than other fungal species. Overall, *Rhizopus* spp were the least detected in all coffee samples assessed (Figure 5).

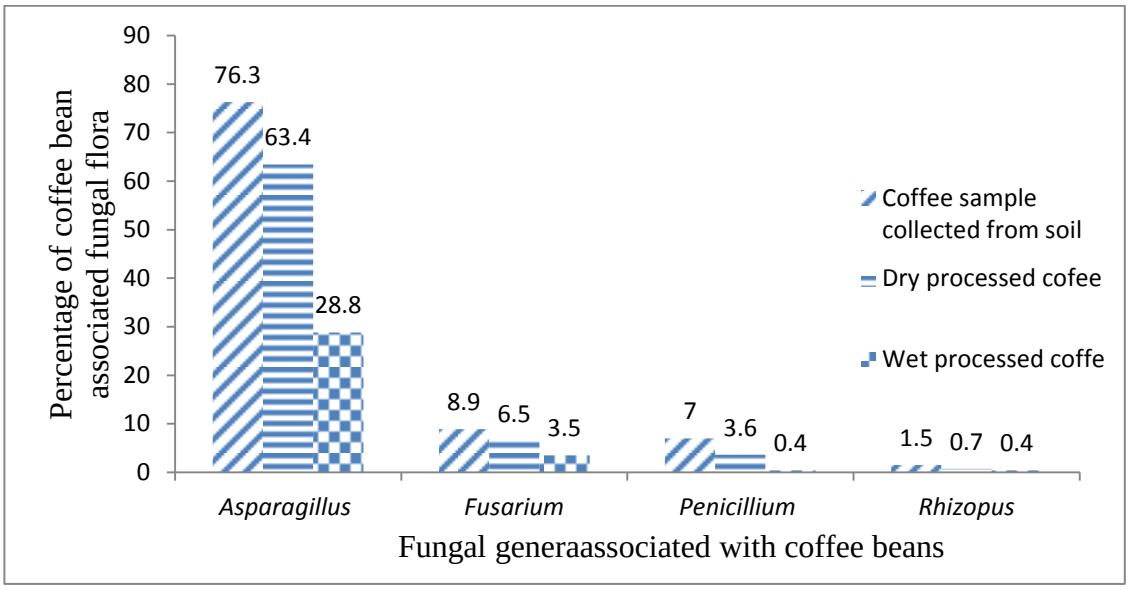


Figure 5.Percentage of total mycoflora associated with coffee collected from soil, dry processed and wet processed sources.

4.3 Cultural and morphological characterization of mycotoxigenic fungi associated with coffee beans

4.3.1 Cultural appearance of mycotoxigenic fungal species associated coffee bean

Mycotoxigenic fungal species associated with coffee beans have different cultural characteristics. The finding of this study indicated that different mycotoxigenic fungal species exhibited different colony colors, margins and different growth rate on different artificial media that Malt Extract Agar(MEA), Czapek Yeast Extract Agar at 25^{o}C(CYA25), Czapek Yeast Extract Agar at 37^{o}C(CYA37), CzapekDox agar (CZA) and Czapek Yeast Extract Agar with 20% sucrose concentration(CY20S) (Fig 7). These mycotoxigenic fungi associated with coffee bean were black *Asprgilil(*section Niger)Yellow *Aspergilli*,(section Cercumdati) and blue to green *Aspergilli (*section Flavi) (Figure 6).

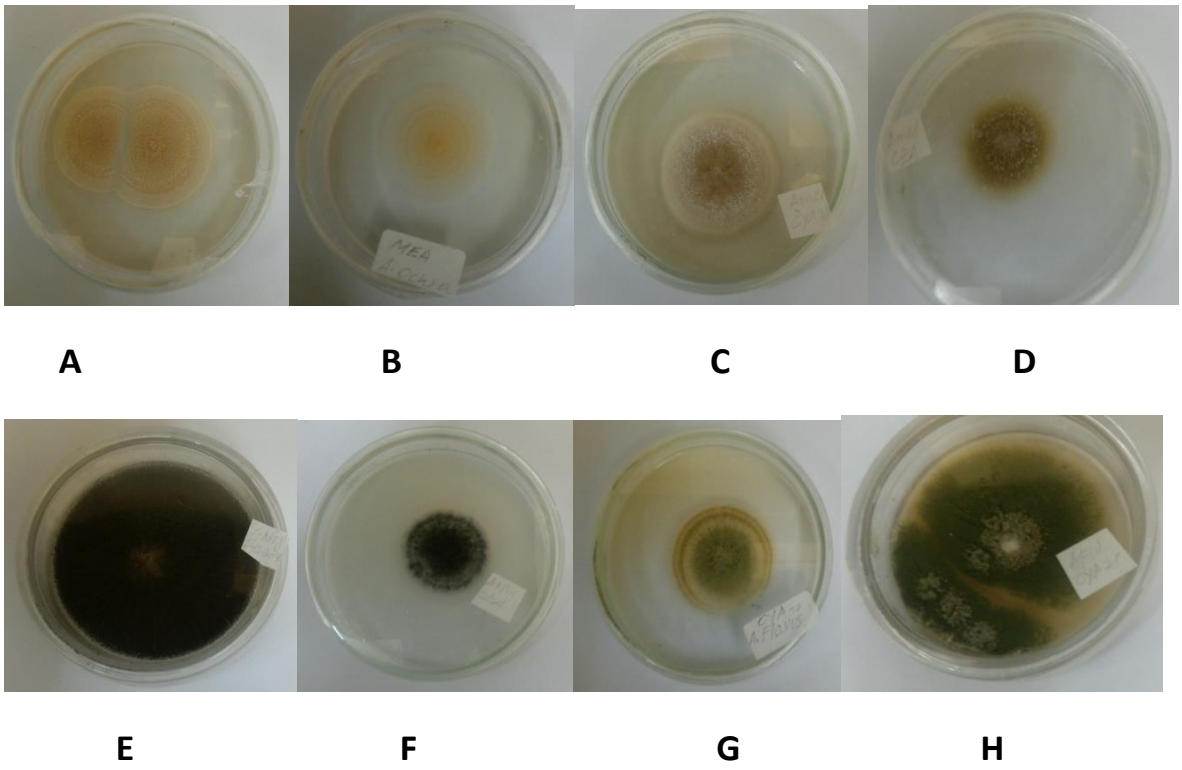


Figure 6. Seven days old colonies on culture media:

Indicates; A. *A. ochraceus* (CYA25); B. *A. ochraceus* (MEA); C.*A. melleus* (CYA25); D. *A. melleus* (CZA); E. *A. niger* (CYA20%) ; F. *A. niger* (CZA); G. *A. flavus* (CYA37); H. *A. flavus* (CYA25).

4.3.2 Colony colors of coffee bean associated mycotoxigenic fungal species on different media

Colony colors of different mycotoxigenic fungi on different culture media indicatedA.niger dark brown on CZA, MEA and CY20S while black color on CYA37 and CYA25, *A.ochraceus* light yellow on MEA and ochraceus on CZA, CYA25, and CY20S, *A.flavus* olive green on CZA, CYA25, CYA20S , olive to olive brown on CYA37 and olive on MEA, *A.melleus* light golden yellow on CZA, CYA25, CYA37 pale yellow on MEA and light yellow on CY20S (Table 3).

Table 3. Cultural characteristics of mycotoxigenic fungi (colony colors) on different media

Colony colors					
CZA	MEA	CYA25	CYA37	CY20S	Species
dark brown	dark brown	Black	Black	dark brown	Black Aspergilli *(A.niger)*
Ochraceus	Light yellow	Ochraceus	No growth	Ochraceus	Yellow Aspergilli *(A.ochraceus)*
Olive green	Olive	Olive green	Olive to olive brown	Olive green	Blue to green *Aspergilli(A.flavus)*
Light golden yellow	Pale yellow	Light golden yellow	Light golden yellow	Light yellow	Yellow Aspergilli (*A.melleus)*

4.3.3 Mycillial colors of coffee bean associated mycotoxigenic fungal species on different media

Table 4. Cultural characteristics of mycotoxigenic fungi (mycelia color) on different media

Mycelium					Species
CZA	MEA	CYA25	CYA37	CY20S	
White	White	White to dull yellow	White to dull yellow	White to dull yellow	Black *Aspergilli (A.niger)*
White	White	White	White	White	Yellow *Aspergilli(A.ochraceus)*
White	White	White	White	White	Blue to green *Aspergilli(A.flavus)*
White to yellow	White to yellow	White to yellow	White to yellow	White to yellow	Yellow *Aspergilli*(*A.melleus)*

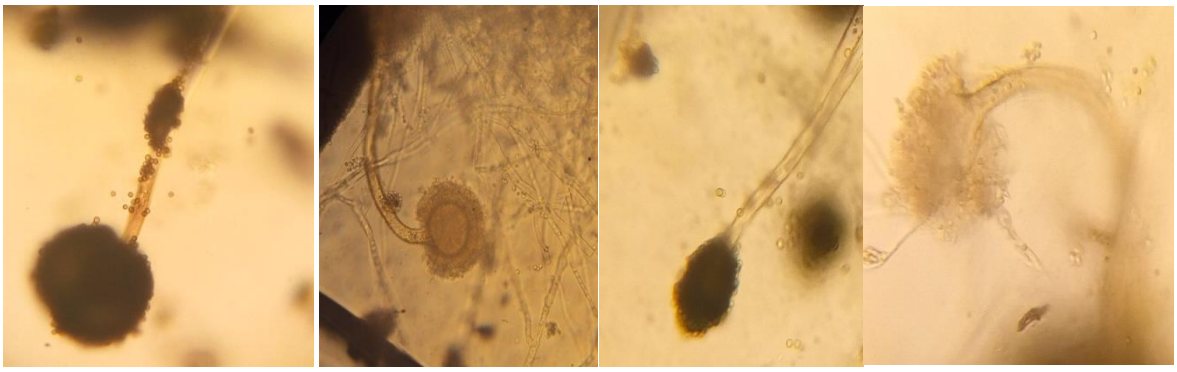
Abbreviations: CZA=CzapekDox Agar, CYA25= Czapek Yeast Extract Agar at 25^{o}C , CYA37= Czapek Yeast Extract Agar at 37^{o}, CY20S= Czapek Yeast Extract Agar with 20% sucrose concentration, MEA=Malt Extract Agar

4.3.4 Stipe length, vesicle diameter and shape, conidia lengh and shape of coffee bean associated mycotoxigenic fungal species

Table 5. Microscopic characteristics of coffee bean associated mycotoxigenic fungi

	Vesicle		Conidia		
Stipe (µM)	Diameter (µM)	Shape (µM)	Length (µM)	Shape (µM)	Species
320-340	32-35	Gl	3-3.5	El	Black *Aspergilli (A.niger)*
400-430	32-35	Gl	3.2-3.8	El	Yellow *Aspergilli (A.ochraceus)*
400-450	45-48	Elon	3.2-3.6	gl/el	Blue to green *Aspergilli(A.flavus)*
350-370	32-37	Sp	2.5-3	El	Yellow *Aspergilli (A.melleus)*

Abbreviations: gl = globose, el = ellipsoidal, py = pyriform, sp = spathulate, elon =elongated



A **B** **C** **D**

Figure 7.Microscopic characters of coffee bean associated fungi

A. *Aspergillus niger* B. *Aspergillus ochraceus* C. *Aspergillus flavus* D. *Aspergillus melleus*

4.4 Fungal species associated with different coffee processing methods and from different coffee sampling method

The finding indicated that four fungal species, including black Aspergilli(*Aspergillus niger),* yellow *Aspergilli(Aspergillus ochraceus),* yellow *Aspergilli (Aspergillus melleus),* andblue to

green *Aspergilli(Aspergillus flavus)* were found associated with coffee beans collected from different coffee processing facilities (wet and dry) and agro ecologies. Out of the four mycotoxigenic fungi, the frequently isolated fungal species was *Aspergillus niger* (69.3%, 54.7%, 26.7%), followed by *Aspergillus ochraceus* (5.6%, 7.7%, 1.8%), *Aspergillus melleus* (1.5%, 0.4%, 0.4%) from coffee samples of soil, dry processed and wet processed respectively (Fig 6).

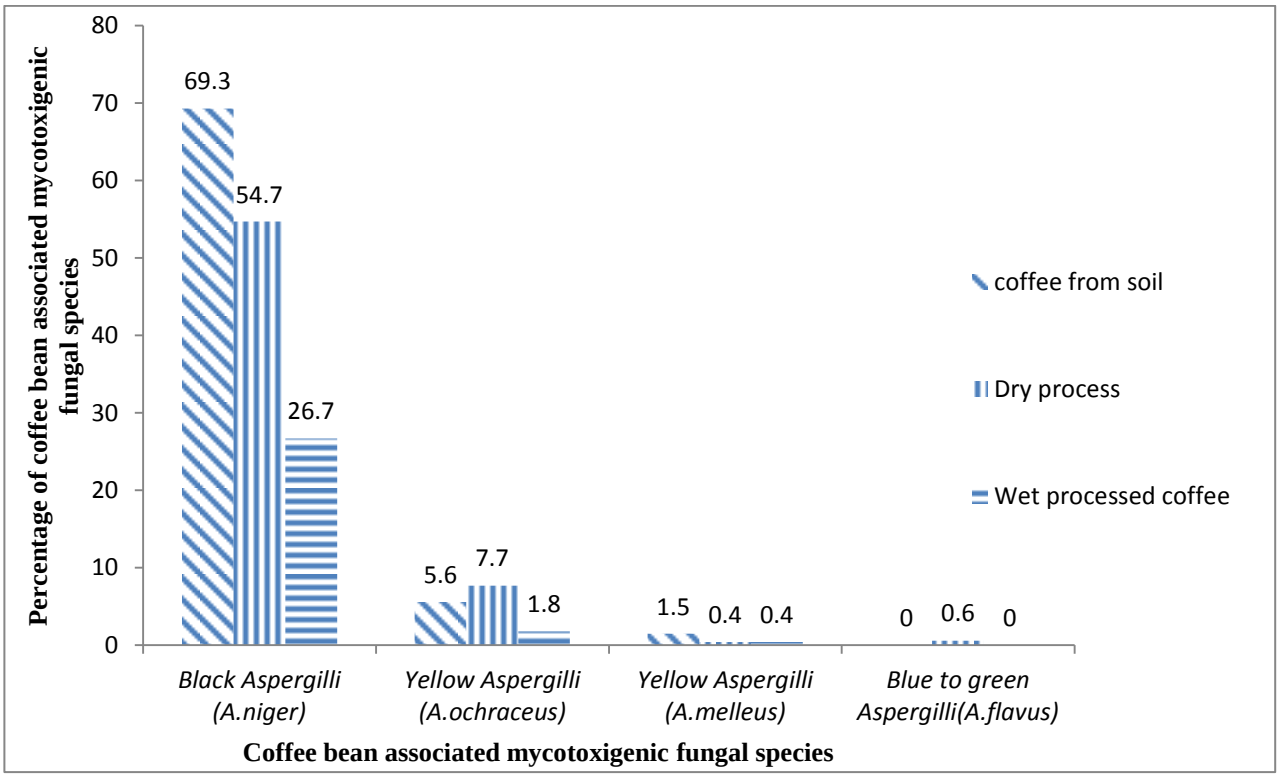


Figure 8. Percentage of mycotoxigenic fungal species associated coffee bean.

4.5 Detection of ochratoxin A from green coffee bean

The maximum OTA detected was 10.61µg/kg from coffee collected from soil and the minimum was ND (not detectable) from both dry processed and wet processed. The mean OTA detected from different coffee samples were; 6.24µg/kg, 2.05µg/kg and 1.2µg/kg from soil, dry processed and wet processed respectively (Table 6). There was high OTA contamination level from samples collected from soil and comparatively less OTA contamination from dry processed while there was least OTA contamination level from wet processed coffee type.

Table 6. Level of detection of Ochratoxin A in different coffee bean samples

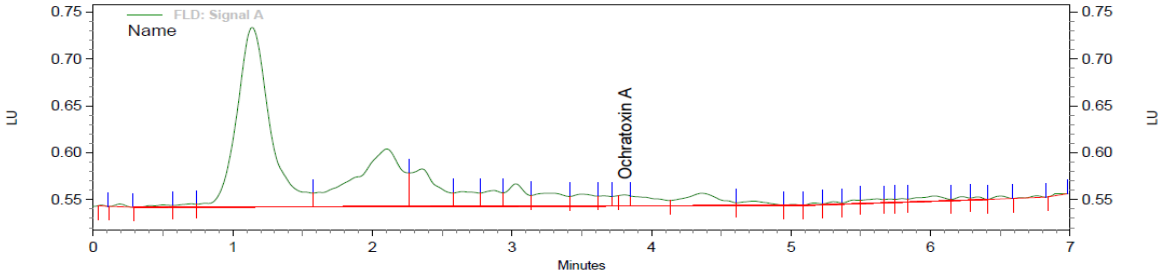
Source of green coffee bean	Sample size	Ochratoxin A detection level (µg/kg)		
		Mean ±sd	Max.	Min
Soil	3	6.24±3.79	10.61	3.88
Dry processing	13	2.05±2.27	8.16	not detectable
Wet processing	4	1.2±0.22	1.82	not detectable
P-value	0.023748			

4.6 Survey result of the study areas

The questionnaire response of assessed coffee growers indicated that as they never used any chemicals for managing diseases while they apply cultural practices and stretching their products over locally prepared bed for dry processed type and over wire mesh. All the assessed coffee growers store their products in sack (*kesha)* and they collect the coffee when matured by hand picking the red cherry from the coffee plants. At harvesting, some coffee may fail to land and they collect from the ground soil and kept separately and finally they use for local consumption and sell locally. All the assessed farmers lack the knowledge of what mycotoxin is and what the cause it has.

4.7 HPLC chromatogram of the test material

The result indicated that the concentration of the loaded green single coffee bean samples to HPLC was OTA concentration of 0.902 ppb with retention time of 3.809 min (Figure 10).



FLD: Signal A Results

Pk #	*Name*	*Retention Time*	*Area*	*Concentration*
14	Ochratoxin A	3.809	9879	**0.902**
Totals			9879	**0.902**

Figure 9. HPLC-FL chromatogram of OTA test material (green coffee).

4.8 Correlation analysis

The correlation analysis among different factors indicated that there was a positive (r=0.527; p=0.04)correlations between Ochratoxin A and moisture content, positive (r=0.802; p<.0001) correlation between OTA and insect damage, positive (r=0.528; p=0.01) correlation between OTA and *A.niger,* positive (r=0.640; p=0.002) correlation between OTA and *A.ochraceus*, positive (r=0.625; p=0.002) correlation between OTA and visible mold overgrowth, while there was no positive correlation of OTA with *A.melleus,* OTA with *penicillium*, OTA, with humidity, OTA with temperature, OTA with altitude (Table 7).

There was also a strong positive (r=0.636; p=0.002) correlation between moisture content and insect damage, positive (r=0.445; p=0.04) moisture content with *A.ochraceus* while there was no positive correlation between moisture content and *penicillium* occurrence, moisture content with *A.melleus*, moisture content with *A.niger*, moisture content with humidity, moisture content with temperature, moisture content with altitude (Table 7). A positive (r=0.454; p=0.04) correlation was observed between insect damage and *A.niger*, positive (r=0.687; p<.001) correlation between insect damage and *A.ochraceus*, positive (r=0.698; p<.001) correlation between insect damage and visible mold overgrowth, while there was no positive correlation among insect damage and A. *melleus*, insect damage and humidity, insect damage and temperature, insect damage and altitude. There was positive correlation (r=0.530; p=0.01) between *A.nge r*and *A. ochraceus*, positive (r=0.546; p=0.01) correlation between *A. niger* with visible mold overgrowth, while no positive correlation between *A.niger* and *penicillium*, *A.niger*and humidiy, *A.niger*and temperature, *A.niger* and altitude. There was a positive (r=0.855; p<.0001) correlation between *A.ochraceus* and visible mold overgrowth (Table 7).

Table 7. Pearson Correlation coefficients among Ochratoxin A, Moisture content, Insect damage, *A.niger*, *A.ochraceus A.melleus*, *Penicillium*, Mold condition, Humidity, Temperature, Altitude

	Ochratoxin A	**Moisture content**	**Insect damage**	***A.niger***	***A.ochraceus***	***A.melleus***	***penicillium***	**Visible mold**	**Humidity**	**Temperature**	**Altitude**
Ochratoxin A		0.527*	0.802***	0.528*	0.640**	0.136^{ns}	0.128^{ns}	0.625**	-0.163^{ns}	0.230^{ns}	-0.174^{ns}
Moisture content	0.527*		0.636**	0.354^{ns}	0.445*	0.112^{ns}	0.226^{ns}	0.388	0.113^{ns}	0.118^{ns}	0.258^{ns}
Insect damage	0.802***	0.635**		0.454*	0.687***	0.235^{ns}	0.439^{ns}	0.698***	0.004^{ns}	0.092^{ns}	0.147^{ns}
A.niger	0.528*	0.354^{ns}	0.454*		0.530*	0.423^{ns}	0.192^{ns}	0.546**	0.186^{ns}	0.184^{ns}	-0.126^{ns}
A.ochraceus	0.640**	0.445*	0.687***	0.530*		0.020^{ns}	0.0234^{ns}	0.855***	0.060^{ns}	0.203^{ns}	-0.120^{ns}
A.melleus	0.136^{ns}	0.112^{ns}	0.235^{ns}	0.423^{ns}	0.020^{ns}		0.107^{ns}	0.154^{ns}	-0.140ns	0.149^{ns}	-0.28^{ns}
Penicillium	0.128^{ns}	0.226^{ns}	0.439^{ns}	0.192^{ns}	0.0234^{ns}	0.107^{ns}		0.103^{ns}	-0.139^{ns}	0.083^{ns}	0.363^{ns}
Visible mold	0.625**	0.388	0.698***	0.546**	0.855***	0.154^{ns}	0.103^{ns}		0.151^{ns}	0.151^{ns}	0.221^{ns}
Humidity	-0.163^{ns}	0.113^{ns}	0.004^{ns}	0.186^{ns}	0.060^{ns}	-0.140^{ns}	-0.139^{ns}	0.151^{ns}		-0.644**	0.286^{ns}
Temperature	0.230^{ns}	0.118^{ns}	0.092^{ns}	0.184^{ns}	0.203^{ns}	0.149^{ns}	0.083^{ns}	0.151^{ns}	-0.644**		-0.497*
Altitude	-0.174^{ns}	0.258^{ns}	0.147^{ns}	-0.126^{ns}	-0.120^{ns}	-0.28^{ns}	0.363^{ns}	0.221^{ns}	0.286^{ns}	-0.497*	

Where: *A.niger=Aspergillus niger, A.ochraceus=Aspergillus ochraceus, A.melleus=Aspergillus melleus* *** Significant at P<0.001, ** Significant at P<0.01, * Significant at P<0.05, ns: no significant difference

5. DISCUSSION

Four major fungal genera *Aspergillus,Fusarium, Penicillium*and *Rhizopus* were detected in association with coffee beans studied in Jimma zone. *Aspergillus* spp were dominantly associating with coffee beans which accounts (84.74%), followed by *Fusarium* (8.75%), *Penicillium* (5.49%) and *Rhyzopus* (1.02%). The results were in line with Geremew *et al.*, (2016) who reported that *Aspergillus* (79%), *Fusarium* (8%), *Penicillium* (5%) found in association with coffee beans. Urbano *et al*.,(2001) have also reported that isolated filamentous fungi in coffee beans from Brazil found *A. ochraceus* and *A. niger*as major contaminants. According to Pitt and Hocking (1997), *Aspergillus* competes for substrate with *Fusarium* and *Penicillium*, and its incidence increases in an environments with high temperature and low water activity, which are the ideal conditions found in the final stages of processing, drying and storage of coffee beans.

Coffee Arabica beans collected from the soil were highly contaminated by four fungal genera followed by dry and wet processed coffee beans. The coffee cherries collected from soil exhibited highest contamination. Since soil serves as a natural habitat for many microorganisms probably due to the fact that coffee cherries remain contact with humid soilby, favoring infection and OTA synthesis by the ochratoxigenic fungi. Urbano *et al*., (2001) have reported that high level of contamination by including *A. ochraceus* and *A. niger*, was observed in raw coffee beans collected in different producing regions of Brazil. Similarly, Abraham (2006) has reported that the association of ochratoxicogenic fungi on Arabica coffee bean samples collected from three localities in south west Ethiopia showed higher incidence of *A. ochraceus* on unwashed coffee dried on bare floor than washed coffee on drying beds.

The results showed that the removal of coffee husk (cherry dehusking) and mucilage (cherry dispulping) by wet processing reduced bean microbial contamination, thus indicating that fungi and OTA contamination would be concentrated in the skin until processing. Likewise, Frank (1999) has reported that pulping would significantly reduce the risk of OTA contamination during subsequent fermentation and drying. Bucheli *et al*. (2000) have also reported that coffee bean skin is the main substrate for the development of ochratoxigenic fungi.OTA accumulated more in dry processed coffee due to the fact that fungi would get

favorable condition biosynthesize OTA in the skin (Batista *et al*., 2009).The high mold contamination observed in dry processed coffee could be attributed to inefficient drying of the cherries. In dry processed coffee, cherries are dried without separation of pulp from the beans. Hence, depending on the weather conditions, the coffee cherries may take long time to dry. Similarly, it may take longer time to dry the whole coffee cherry compared to the parchment coffee (Bucheli and Taniwaki, 2003).

The results revealed that samples from soil were highly contaminated with OTAcompared to dry and wet processed coffee beans. The present study indicated that the concentration of OTA ranges from not detected (ND) to 10.61 µg/kg. A fraction of coffee swept from ground is composed of fruits displaying a larger number of defects and, consequently, has a greater risk of OTA contamination. The result of the current study is in agreement with Moraes and Luchese (2004) who observed a higher concentration of OTA in coffee samples dried on ground coffee yards than in those dried on cement coffee yards. According to the authors, coffee processing on cement platform is important in preventing OTA contamination, while coffee beans processed in ground coffee yard are more favorable to fungal contamination. Our result also supported by Paterson *et al*., (2014) who reported that coffee beans obtained after falling on the soil were highly contaminated with OTA.

Culliao and Barcelo, (2015) have reported that OTA was more commonly detected in Robusta coffee (37%) than in Arabica coffee (26%). The highest (97 µg/kg) level of OTA found in the dried whole cherries of Arabica, while for Robusta, the (120 µg/kg) highest level of OTA was found. Romani, *et al.*,(2000) also reported that the detected OTA from green coffee beans was 18–48 µg/kg in Congo. Geremew *et al., (*2016) also reported that mean OTA concentration from locally consumed Ethiopian coffee was 1.5µg/kg.

In Ethiopia, the coffee collected from ground soil at the end of harvesting time is consumed at home. The coffee that is collected from ground soil exhibited relatively high concentration of OTA and frequently consumption of such coffee may be a risk for Ochratoxin A accommodation in the body of the consumer. It has to be managed since it causes detrimental effect on coffee quality and health effect to human being. Ochratoxin A is primarily a kidney

toxin but in sufficiently high concentrations it can damage a liver as well. Ochratoxin is a carcinogen in rats and mice and is suspect as the causative (carcinogenic) agent of human disease. Balkan Endemic Nephropathy is one such kidney disease, often with associated tumors, of humans that is considered by some to be caused by ochratoxin (Pfohl- Leszkowicz *et al*., 2002). According to Simon (1996) epidemiologic studies showed that in areas where high OTA levels are reached in food and in the blood of the population, there is a high incidence of nephropathy and renal tumors.

The current study revealed that when toxigenic strains of *Aspergillus ochraceus* and *Aspergillus niger* had positive significant correlation with OTA. This shows that the presence of toxigenic strains involves a great risk of OTA presence when it gets favorable conditions, these mycotoxigenic fungi have a potential to produce high amount of OTA. The study of Taniwaki *et al*., (2003) reported that the isolated amount of mycotoxigenic fungi 75% of *Aspergillus ochraceus* and 3% of *Aspergillus niger* were OTA producers.

The correlation result indicated that there was strong positive correlation between OTA concentration, coffee berry borer (CBB) damage, moisture content, mold contamination. Coffee berry borer are common around coffee fields so that they penetrate the coffee berry and allow it to moisturize so that the fungi can easily colonize the berries. Moreover, CBB can act as a vector for the transmission of mycotoxigenic fungal spores. This result was supported by Vega and Mercadier (1998) who reported that presence of OTA in insect damaged type of sample may be due to injuries caused by *Hypotenemus hampei* Ferrari, known as the coffee berry borer which is a vector of *A. ochraceus*.Velmourougane, *et al*., (2010) also reported that higher microbial contamination was observed in coffee berry borer infested beans in both the varieties of arabica and robusta coffee with the presence of toxigenic molds (such as *Aspergillus niger* and *Aspergillus ochraceus*). The current study indicated that the altitudinal variation showed no correlation with the ochratoxin A concentration. The concentration of OTA was highly correlated with coffee processing methods and with samples from ground soil.

6. CONCLUSION

In conclusion, coffee is contaminated by various filamentous molds during postharvest phases. *Aspergillus* spp were dominant fungi associated with coffee beans that had contact with soil following *Fusarium* spp, *Penicillium* spp and *Rhizopus* spp.

The highest ochratoxin A level (10.61µg/kg) was found from coffee beans collected from soil samples while the least ochratoxin A (Not detectable) were found from both dry processed and wet processed coffee. The mean OTA level was 6.24, 2.05, and 1.2 from soil, dry processed and wet processed coffee beans, respectively. CBB, beans moisture content, and visible mold overgrowth are major factors that favor colonization of coffee beans with mycotoxigenic fungi and OTA contaminations.

7. RECOMMENDATIONS

- Avoiding collecting coffee from soil.
- Insect pest infestation in the field as well as in the postharvest stage needs to be controlled since insects used as a vector and also the damaged seeds provide point of entry that enhance mold growth.
- Coffee beans need to be dried to proper moisture level (8-12%) before storage to reduce mold development.
- Avoiding drying over soil ground as it increase mold formations.
- Shifting to wet processing methods since it is less susceptible for mold colonization.
- Awareness should be created for the farmers and all stakeholders about mycotoxinogenic genera and mycotoxins they produce.
- Further study should be conducted on all major coffee growing areas of Ethiopia.

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9. APPENDICES

Appendix 1.A questionnaire developed for collecting data of coffee bean for the study of mycotoxigenic fungi and their mycotoxins

Country Ethiopia Region Oromia Zone Jimma

District Peasant association ----------

Farmer's name ---------- Location ----------

Altitude…..... Longitude ….....Latitude.....

1. Name of the cultivar they use_____

A. improved ( ) B. local

2. Coffee production system they used

A .Forest B. semi forest C. garden D. plantation

3. Diseases management practice they used in the field

A. applying pesticide B. Cultural management

4. Method of harvest ----------

A. Hand picking from the tree B. collecting from land

5. Time of harvest ----------

A. early stage B. matured stage C. after defoliated to the land

6. What type of drying system they use to dry their grains after harvesting?

A. On bare soil B. over polyethylene C .over korkoro

7. For how long time crops dries before storage after maturity

A. for a week B. from week up to a month C. above a month

8. Storage place used by farmers

A. underground B. in locally prepared pit C. in sack

9. How do farmers store their new products in the storage?

A. They don't mix them with previous products B. They mix them with last year stored products C. They store different grain in the same storage

10. Storage time

A. for a month B.1-2 months C. 2-4 months D. above 4 months

11. Estimated yield loss by postharvest fungi contaminations

A.1-5 % B.6-10% C.10-20% D. above 20%

12. What farmers do if their grains are contaminated with mould?

A. they separate and dispose contaminated one B. they give to animals C. they feed themselves

13. Do farmers have knowhow about mycotoxins and their negative effects?

A. yes B. no

Appendix 2. Different media formulations

Czapek concentrate

Ingredients gram/Liter

$NaNo_3$ 30.0g

Kcl.....5.0g

$MgSO_4.7H_2O$.....5.0g

$ZnSO_4.7H_2O$.....0.1g

$CuSO_4.5H_2O$.....0.05g

$FeSO_4.7H_2O$.....0.1g

Czapek Yeast Agar (CYA25, 37)

Ingredients gram/Liter

K_2HPO_4.....1.0g

Czapek concentrate.....10.0ml

Powdered Yeast Extract.....5.0g

Sucrose.....30.0g

Agar.....15.0g

Distilled water.....1000ml

Czapek Yeast Agar with 20% sucrose (CY20%S)

Ingredients gram/Liter

K_2HPO_4.....1.0g

Czapek concentrate.....10.0ml

Powdered Yeast Extract.....5.0g

Sucrose.....200.0g

Agar.....15.0g

Distilled water.....1000ml

Malt Extract Agar

Ingredients gram/Liter

Powdered Malt Extract.....20.0g

Poptone.....1.0g

Glucose.....20.0g

Agar.....20.0g

Distilled water.....1000ml

CzapekD Agar (CZA)

Ingredients gram/Liter

K_2HPO_4.....1.0g

Czapek concentrate.....10.0ml

Sucrose.....30.0g

Agar.....17.50g

Distilled water.....1000ml

Appendix 3. Calibration Report of The standard OTA solutions

Method: D:\Mycotoxins\Ochratoxin A\Method\Determination of Ochratoxin A (20190627).met

Print Time: 4/7/y19 12:32:04 PM (GMT +04:00)

User: SYSTEM (SYSTEM)

Instrument: HPLC

Ochrato2xin A (FLD: Signal A)

Average RF: 9.03717e-005 RF StDev: 2.07276e-006 RF %RSD: 2.29359

Scaling: None LSQ Weighting: None Force Through Zero: On

Replicate Mode: Replace

Fit Type: Linear

y = 9.12747e-005x + 0.000000

Goodness of fit (r^2): 0.997800

Peak: Ochratoxin A -- ESTD -- FLD: Signal A

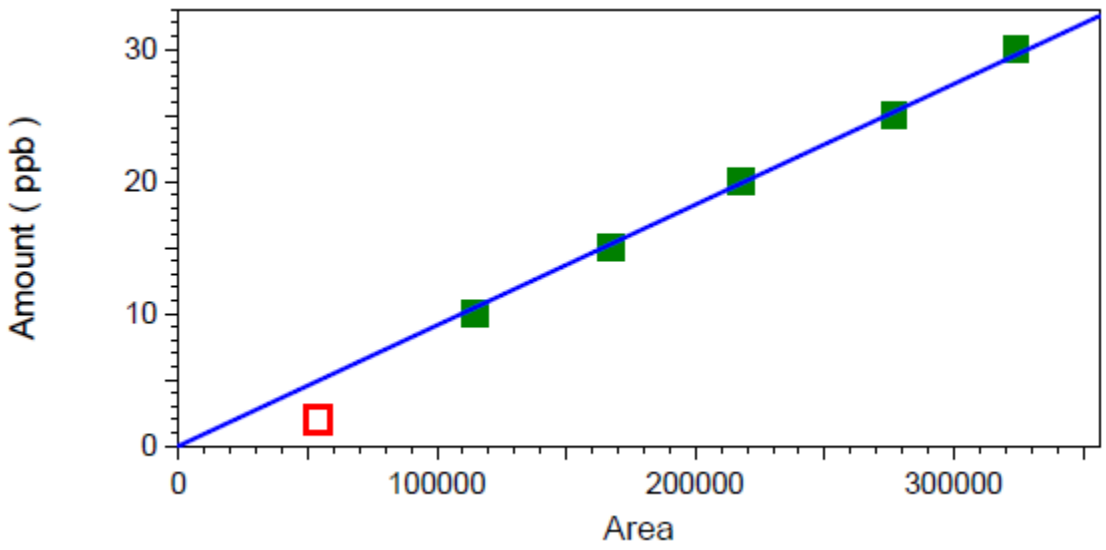

Figure 10.Calibration curve of standard solution

	Level 1	Level 2	Level 3	Level 4	Level 5
Amount	2	10	15	20	25
Area	54007*	114531	167206	217551	276832
RF	3.703223656 19272e-005	8.73126053208 302e-005	8.970969941270 05e-005	9.193246641017 51e-005	9.0307478904 1729e-005
Last Area Residual	-2.92947	-0.453786	-0.261682	0.143092	-0.267765
Rep StDev					
Rep %RSD					
Rep 1 Area	54007*	114531	167206	217551	276832
Rep 1 User	SYSTEM	SYSTEM	SYSTEM	SYSTEM	SYSTEM
Rep 1 Data File	D:\Mycotoxins\Ochratoxin A\Result\2019-07-02 11-37-58 (GMT +04-00)2 ppb Ochratoxin A.rslt\2 ppb Ochratoxin A-Rep2.dat	D:\Mycotoxins\Ochratoxin A\Result\2019-07-02 10-29-02 (GMT +04-00)10 ppb Ochratoxin A.rslt\10 ppb Ochratoxin A-Rep1.dat	D:\Mycotoxins\Ochratoxin A\Result\2019-07-02 10-29-33 (GMT +04-00)15 ppb Ochratoxin A.rslt\15 ppb Ochratoxin A-Rep2.dat	D:\Mycotoxins\Ochratoxin A\Result\2019-07-02 10-30-13 (GMT +04-00)20 ppb Ochratoxin A.rslt\20 ppb Ochratoxin A-Rep3.dat	D:\Mycotoxins\Ochratoxin A\Result\2019-07-02 10-30-43 (GMT +04-00)25 ppb Ochratoxin A.rslt\25 ppb Ochratoxin A-Rep3.dat
Rep 1 Sample ID	2 ppb Ochratoxin A	10 ppb Ochratoxin A	15 ppb Ochratoxin A	20 ppb Ochratoxin A	25 ppb Ochratoxin A
Rep 1 Calib. Time	2/7/y19 11:38:01 AM (GMT +04:00)	2/7/y19 10:29:04 AM (GMT +04:00)	2/7/y19 10:29:35 AM (GMT +04:00)	2/7/y19 10:30:16 AM (GMT +04:00)	2/7/y19 10:30:45 AM (GMT +04:00)