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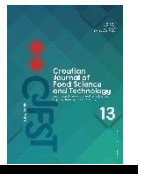

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Aflatoxins and aflatoxigenic fungi in export standard white and red sesame seeds (*Sesamum indicum L.*) from Ethiopia

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ABSTRACT

The oil seed sectors one of the fastest growing sectors in Ethiopia, both in terms of its foreign exchange earnings and as a source of income for millions of Ethiopians. Oilseed crops are recognized to be potentially suitable substrate for the production of aflatoxins by aflatoxigenic fungi. This study intended to evaluate knowledge, attitude and practice of sesame exporters about aflatoxins contamination, to characterize the seed and to determine the level of aflatoxins, from export standard white and red Humera and Wollega sesame seeds. Twenty seven ready to export sesame samples were collected from Ethiopian Commodity Exchange (ECX) warehouse. Thousand seed weight, moisture, crude fat and peroxide value were determined according to AOAC. Aflatoxin B1, B2, G1, G2 and total aflatoxins were determined at ng/g level by Supel™ Tox Aflazea cartridge clean up and reversed-phase liquid chromatography. Thousand seed weight determined in the range of 2.89-3.22 g/1000 seeds, moisture is 3.33-4.99%, crude fat is 46.75-57.75% and peroxide value is 2-8.2 meq/Kg. Aflatoxin B1, B2, G1, G2 and total aflatoxins detected in the range of 1.32 - 2.12 ng/g, 1.52-31.98 ng/g, 16.2-48.28 ng/g, 0.56-9.04 ng/g and 0.44-64.96 ng/g, respectively. The aflatoxins contamination of sesame seeds could ruin the oilseed export and thus decrease the revenues of the state.

Introduction

Sesame *(Sesamum indicum* L.*)* is an important oil seed crop belonging to the family *Pedaliaceae*. It grows in tropical zones as well as in temperate zones between latitudes of 40ºN and 40ºS (Onsaard, 2012; Ogbonna and Ukaan, 2013). Ethiopia is among the top six producers of sesame seed in the world (Wijnands et al., 2009). Geographically, sesame is produced in different parts of Ethiopia at an elevation from sea level of about 1500 meters. The dominant producers, who contribute over 83 percent to national production, are located in the regions of Tigray (West Tigray), Amhara (Humera), Wollega and most recently, in Benishangul-Gumuz Region (Metekel) (CSA, 2010).

Oilseed crops are recognized to be potentially suitable substrates for the production of toxic secondary metabolites by molds, notably the production of aflatoxins by toxigenic strains of *Aspergillus flavus* and *Aspergillus parasiticus* (Hesseltineet al., 1966). The fungi from the genus *Aspergillus*, especially *A. flavus* and *A. niger*, are fungal species that often cause problems in oilseeds related to discoloration, rotting, seed shrinking, necrosis, germination and endup with aflatoxins production Chavan and Kakde, 2008). Furthermore, lipid oxidation of oilseeds increases the biosynthesis of aflatoxins (Fanelli and Fabbri, 1989; De Luca et al., 1995; Jayashree and Subramanyam, 1999; Bircan, 2006).

Studies on occurrence of aflatoxins on sesame seeds were done in different countries of the world. In Greece, thirty samples of sesame products were analyzed for the presence of AFB1. Out of them, 77.6% were contaminated and in eight AFB1 concentration exceeded the maximal tolerable limit of European Union (EU) (2 ng/g) (Eleniet al., 2016). In Nigeria also eight sesame samples out of thirty contained AFB1 within the range of 14.71 to 140.9 ng/g (Makunet al., 2014). These results

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indicate that aflatoxin contamination in sesame was found to be beyond the tolerable limit set by the European Commission and require similar work in Ethiopia.

There are numbers of studies conducted in Ethiopia by different authors on aflatoxin contamination of different agricultural commodities, foods and feeds like groundnut (Eshetu, 2010; Ephrem et al., 2014; Mohammed and Chala, 2014), maize (Ayalew, 2010), sorghum, milk, shiro and ground red pepper (Fufa and Urga, 1996; Ayalewet al., 2006). A study by Ayalew et al. (1995) reported aflatoxin levels of 5-250 ng/g in groundnut seed from eastern Ethiopia (Ayalewet al., 1995). Similarly, the work by Alemayehu et al. (2012) reported extremely high levels of total aflatoxin contamination in *Aspergillus flavus* positive samples of groundnut seed that varied between 15 and 11865 ng/g (Alemayehu et al., 2012). These studies showed that groundnut samples from Ethiopia are contaminated at levels much higher than any international standard or recommendation and that the contamination is far beyond Food and Agricultural Organization of the United Nations (FAO), World Health Organization (WHO) standard (15 ng/g) and the European Union (EU) limit, i.e. 4-15 ng/g.

Even though, a number of studies have been conducted in Ethiopia on aflatoxins occurrences in different agricultural commodities, foods and feeds. There are limited studies conducted on aflatoxin contamination of sesame seed and no work has been reported on export sesame seed so far. So, this study was conducted to investigate aflatoxins in export standard white and red sesame seeds from Ethiopia.

Materials and methods

Sample and sampling

Twenty seven ready to export sesame seed samples were collected in accordance with the objective of the study from Ethiopian Commodity Exchange (ECX) Warehouse, locatedin Addis Ababa. ECX is established in 2008 to undertake grading and auctioning of different agricultural commodities including sesame for exporters. According to ECX classification, export standard sesames were categorized into three groups. These are white Humera, white Wollega and red sesame, regardless of different areas where the sesame grown. Consequently, nine samples from white Humera sesame, nine white Wollega sesame and nine red sesame seeds were collected in polyethelene plastic bag by taking preventive measures to avoid contamination. The seeds were separated from foreign matter, milled and sieved to passthrough 1 mm mesh size. The flour packed in tight polyethylene bags and stored in cool dry place until the analysis.

Chemicals

HPLC grade Acetonitrile and methanol, N-hexane, deionized water and pure aflatoxins standards of B1, B2, G1 and G2 obtained from Sigma-Aldrich (St. Louis, MO, USA) and petroleum ether, glacial acetic acid, chloroform, potassium iodide.

Study design for knowledge attitude and practice (KAP)

A total of 30 sesame seed exporters participated in the survey. Purposive sampling technique and semi-structured questionnaires were used to get more information. The questionnaire evaluation was carried out to evaluate knowledge, attitude and practice of sesame exporters regarding food quality and safety issues.

Seed characteristics

Thousand seed weight

Thousand seed weight was determined by using electronic grain counter (Numigral, CHOPIN). The mass of 1000 seeds were measured on electronic balance (ISTA, 1985).

Moisture content

The moisture content of sesame seed was analyzed by using drying oven (Model: DHG-9055A: 2007, Shanghai China). The analysis has been conducted according to (AOAC, 1995).

Crude fat (%)

Crude fat/oil content of sesame seeds was determined by Soxhlet extractor (Model: EV 16, SN: 4002824, Germany), according to (AOAC, 1995).

Peroxide value analysis

Peroxide value of the sesame seed was analyzed according to (AOAC, 1995).

Procedure for aflatoxin analysis

Mobile phase

The mobile phase was water-methanol-acetonitrile (60:25:15, v/v/v) and isocratic method was employed. The mobile phase was filtered by applying vacuum in a filter unit and degassed before use.

Method validations

To evaluate the analytical performance of the instrument and validity of the method identification, accuracy, precision, linearity, asserting working grange, LOD and LOQ were done.

Standard preparation

For method validation, a series (2, 5, 10, 20, 30, 50, 100 and 250 ng/g) of mixed aflatoxins standards were prepared from the stock aflatoxins standard (B1, B2, G1 and G2). Standard solutions were prepared in a 10ml volumetric flasks by using HPLC grade methanol as a diluent.

Sample extraction

Twenty five grams of finely grounded sesame seed samples were accurately weighed and added into 500mL Erlenmeyer flask. One hundred millilitre of extraction solvent (84:16 acetonitrile: deionized water) was added into the sample in the flask sealed with stopper and parafilm. Then, the contents were blended for 3 minutes by using blender. Finally, the suspension was filtered under vacuum until the liquid is filtered by Buchner funnel with filter paper in an Erlenmeyer flask with side arm and a neoprene filter adapter. Filtered extracts were transferred into sample jar and covered with lid until the final purification.

Sample purification

Purification of the sample was done by assembling a Supel™ Tox Aflazea purification cartridge with a samples collecting tube. The extracts (2 ml) collected in the tube were further purified by using 0.45µm micro filter. Finally, neat solution was transferred into 2 ml glass vial and 20µL was injected into HPLC system.

HPLC determination and calculations

Chromatographic system

Shimadzu HPLC instrument with auto sampling system, fluorescence detector and lab solution software were used for analysis. A Shim-pack FC-ODS column (5µm, 250 x 4.6mm diameter) at 25ºC temperature and 1.2ml/min flow rate was used. The running time was 25 minutes, injection volume 20µl, diluent methanol and needle wash (Water: Methanol 90:10 v/v). Aflatoxins were detected at 365 nm excitation and 440 nm emission wavelengths.

Calculations

The elute aflatoxins (B1, B2, G1 and G2) were determined at ng/g level and calculated according to the equation below.

$$\text{Final aflatoxin in ng/g} = m_a \left(\frac{Vf}{Vi}\right) * \left(\frac{1}{mt}\right) \quad (1)$$

Where:
m_a= Aflatoxin level in ng corresponding to the area or height of peak of the sample elute.
V_f= Final test solution elute volume in (µl)
V_i= Volume of elute injected into HPLC in (µl)
m_t= Weight of commodity represented by the final extract in (g)

Isolation and identification of fungi

Twenty sesame seeds per sample were sterilized with 10% sodium hypochlorite solution for 1 min, and immersed into sterile distilled water for 1 min. Seeds were placed in freshly prepared Potato Dextrose Agar (PDA) plates and incubated for three days at 25°C. Pure cultures of different outgrowing fungi were obtained by transferring fungal colonies to new PDA plates by using sterile toothpicks, and the plates were incubated for 5-7 days at 25°C. Isolates were identified to a species level based on morphological (phenotypic) features as described by Mohammed A. and Chala A. (2014).

Experimental data analysis

After collecting the results, all the data entered into IBM SPSS version 20.0 and the required statistical analysis was calculated. The results were reported as percentage and mean ± SD. The Least Significant Difference (LSD) was utilized for mean separation and $P < 0.05$ was considered to be significant.

Results and discussion

Knowledge attitude and practice survey

Sixty percent of sesame seed exporters, who participated in this survey, do not have any idea about aflatoxins and 90% of the respondents cannot know that aflatoxins can contaminate sesame seed. From the total participants of the survey, 97% do not know that improper seed drying and well aerating of sesame storage area is associated

with aflatoxins contamination. Additionally, almost all of the participants do not know that contamination of sesame seed with molds may reduce its overall market acceptability. Practice survey of the participants shows that the respondents cannot apply any method that minimizes aflatoxins contamination of sesame seed. Finally, all of them replied that they cannot conduct any kind of test for the presence of aflatoxins on sesame seed before exporting the seed. The survey finding depicts that there is knowledge, attitude and practice gap on the sesame seed exporters regarding aflatoxin related food safety and quality issues.

Thousand seed weight

Three sesame types, white Humera, white Wollega and red sesame, have an average 1000 seed weight of 3.07 ± 0.39g/1000 seeds (mean ± SD). There is a significant difference ($p < 0.05$) on thousand seed weight of red and the white Humera and white Wollega sesame seeds. The highest 1000 seed weight is for red sesame (3.22g/1000) seeds and the lowest for white Humera type (2.89g/1000) seeds. This result is comparable to the finding reported by Zebib et al. (2015) on physico-chemical properties of sesame varieties grown in northern area of Ethiopia, which is within the range of 2.74-3.16g/1000 seed (Zebib et al., 2015). According to Eckey, (1954) the variation on the thousand seed weight is due to variation in variety and agricultural conditions (Eckey, 1954).

Moisture content of sesame seed

Moisture is one of the main factors that determines the occurrence of aflatoxigenic fungi and aflatoxins production on a certain agricultural commodity or substrate. Average moisture requirement for a feed above 14% may favour toxin production, but the moisture requirement may vary depending on the type of the commodity. For oil-rich nuts, moisture requirement for toxin production is 9-10% (WHO, 1979). The average moisture content of white Humera, white Wollega and red sesame is 4.21 – 5.66%, 3.44 – 4.0% and 3.44 – 4.44%, respectively. These moisture levels are below the average moisture requirements for toxin production on oil-rich nuts. In the present study, higher moisture content recorded as compared to the study finding by Zebib et al. (2015), they reported the moisture content for different types of sesame in the range of 3.17-3.96 %.

Crude fat content of sesame seed

Crude fat/oil content of the oilseeds is the main feature that differ oilseeds from other types of seeds. Improper storage condition makes the oils susceptible to oxidative rancidity, which deteriorates the seed easily and facilitates infestation of the seed by aflatoxins producing molds. Sesame seeds contain higher amount of oil. The average crude fat content of white Humera, white Wollega and red sesame sesame is 49.5-52.0%, 52.25-57.75%, and 46.75-48.75%, respectively. White Wollega sesame seed shows higher amount of oil content as compared to others. The finding of this study is in parallel to the result by Zebib et al. (2015), crude oil in the range of 50.88 - 52.67%. Similarly, Nzikou et al. (2009) also reported 54% average crude fat content of sesame seed from Congo-Brazaville.

Peroxide value of sesame seed

Peroxide value indicates the ability of oil to get rancid due to oxygen absorption during storage and processing. Thus, sesame with high peroxide value have poor resistances to peroxidation. White Humera sesame peroxide value is in the range of 3-4.8 meq/kg, white Wollega sesame 4.4-8.2 meq/kg and red sesame is 2-6 meq/kg, which indicates that the oil of sesame seeds is resistant for oxidative rancidity. This is mainly associated with its antioxidant content like sesamin, sesamol and sesamolin (Elleuch et al., 2007).

Aflatoxins content of sesame seed

Chromatographic method validation

Identification

The identification of four aflatoxins from the test sample has been done based on their retention time from the HPLC chromatogram. As indicated below in Figure 1, the chromatogram obtained by running 30 ng/g mixed aflatoxins standard.

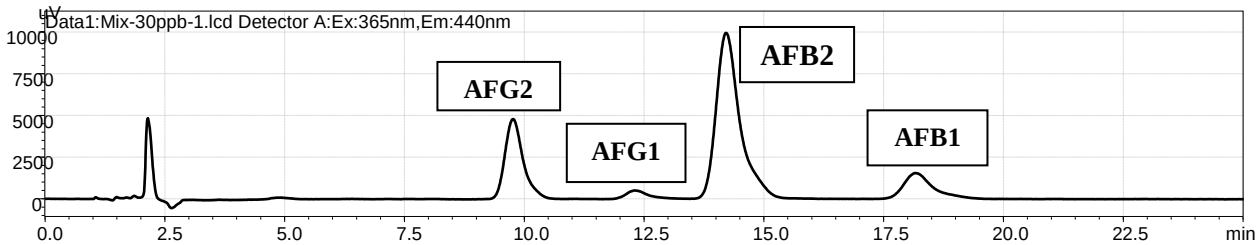

Figure 1. Aflatoxins mixed standard 30 ng/g chromatogram

Table 1. Aflatoxin identification retention time

Aflatoxins	Aflatoxin 250 ng/g injection retention time (min)		N	Mean	Standard Deviation	% RSD
	For single run	For mixed run				
AFG2	9.781	9.76	2	9.77	0.009	0.092
AFG1	12.35	12.30	2	12.32	0.033	0.267
AFB2	14.30	14.21	2	14.26	0.062	0.434
AFB1	18.21	18.165	2	18.18	0.034	0.186

Table 2. Limit of detection and limit of quantification

Aflatoxins	LOD (ng/g)	Signal to noise ratio (S/N)	LOQ (ng/g)	Signal to noise ratio (S/N)
AFG2	0.01	4.152	0.05	10.305
AFG1	1	3.185	2	12.441
AFB2	0.01	8.028	0.05	14.295
AFB1	0.8	7.920	1	11.620

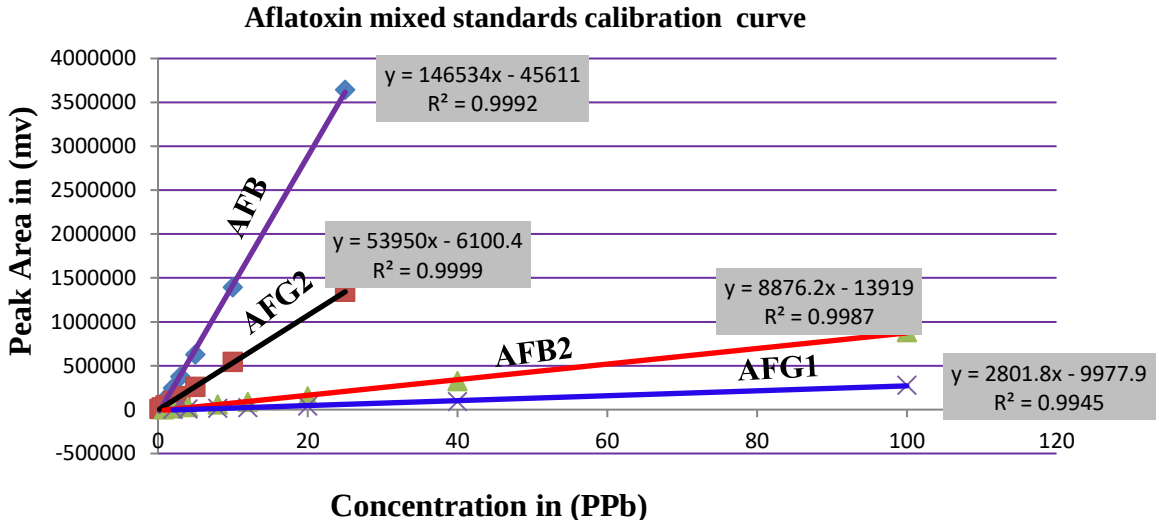

Figure 2. Standard calibration curve for aflatoxin B1, G2, B2 and G1

Percent of relative standard deviation is used to evaluate precision of the retention time. For this purpose, 250 ng/g mixed aflatoxins standard was injected into the HPLC system. As summarized in Table 1. Percent relative standard deviation obtained in the range of 0.092-0.0434%. According to FDA, percent relative standard deviation below 2% is acceptable for the precision test of aan assay (FDA, 2002).

Limit of detection and quantification

Limit of detection (LOD; ng/g) and the limit of quantification (LOQ; ng/g) mean relative standard deviation (% RSD) for the investigated matrix were described in Table 2.

Linearity check

According to the International Conference on Harmonization (ICH) guidelines, a minimum of five concentration levels, along with a certain specified ranges, is recommended for the linearity check up. Accordingly, coefficients of determination ranged from 0.994 to 0.999 for all aflatoxin standards, indicating good linearity and the standard calibration curves was depicted in Figure 2.

Accuracy and recovery test

To check the accuracy of the solid phase extraction (SPE) used for sample purification, recover test was conducted by spiking known amount of analyte (50 ng/g and 100

ng/g) with sesame seed sample extract and the percent recovery of the analyte is calculated. According to FDA, (2002), acceptable level of mean percent recovery is within the range of 100 ±20 or the percent recovery lay within the range of 80-120. As presented below in Table 3 the percentage of recovery determined in the range of 86-97%. This indicates that the method is accurate within the desired recovery range set by FDA and the percent relative standard deviation in less than 5 (FDA, 2002).

Table 3. Statistics for aflatoxin recovery test

Aflatoxins	Spiking Concentration		% Recovery		N	Mean	Standard deviation	% RSD
	100 ng/g	50 ng/g	100 ng/g	50 ng/g				
AFG2	10ppb	5ppb	94.4	95.33	4	94.865	0.657	0.69
AFG1	40ppb	20ppb	83.07	88.72	4	85.895	3.995	4.65
AFB2	10ppb	5ppb	95.22	99.05	4	97.135	2.708	2.77
AFB1	40ppb	20ppb	90.6	88.0	4	89.321	1.838	2.05

Table 4. Sesame sample aflatoxin content (ng/g)

Sample	AFG2	AFG1	AFB2	AFB1	Total Sum
H-1	ND	ND	7.08	1.44	8.52
H-2	ND	ND	7.24	1.32	8.56
H-3	0.64	ND	7.20	ND	7.20
H-4	1.40	ND	ND	ND	1.40
H-5	0.68	ND	ND	ND	0.68
H-6	1.28	16.2	11.2	1.36	30.04
H-7	0.56	34.68	ND	ND	35.24
H-8	1.32	31.76	7.44	ND	40.52
H-9	3.36	ND	ND	ND	3.36
Mean AF	**1.02^{b}**	**9.18^{a}**	**4.46^{a}**	**0.45^{a}**	**15.05^{a}**
R-1	2.08	18.44	ND	1.52	22.04
R-2	1.64	31.92	7.8	1.36	42.72
R-3	2.44	32.92	ND	ND	35.36
R-4	ND	ND	0.44	ND	0.44
R-5	1.24	30.68	10.48	1.48	43.88
R-6	9.04	48.28	31.96	1.52	90.8
R-7	3.24	46.4	15.32	ND	64.96
R-8	ND	ND	ND	ND	ND
R-9	ND	ND	1.72	ND	1.72
Mean AF	**2.18^{b}**	**23.18^{a}**	**7.52^{a}**	**0.65^{a}**	**33.54^{a}**
W-1	1.16	38.08	18.88	2.12	60.24
W-2	1.08	31.92	25.16	1.44	59.6
W-3	ND	ND	ND	ND	ND
W-4	ND	ND	ND	1.62	1.62
W-5	0.68	37.12	14.4	1.64	53.84
W-6	ND	23.72	ND	1.36	25.08
W-7	ND	22.56	8.88	1.48	32.92
W-8	ND	21.36	7.56	1.36	30.28
W-9	ND	< LOD	1.52	1.52	3.04
Mean AF	**0.32^{a}**	**19.41^{a}**	**8.48^{a}**	**1.39^{b}**	**29.62^{a}**
Average total aflatoxin content of Sesame seed is 26.07 ng/g (ppb)					
ND- Not detected, <LOD- below Limit of detection					

Aflatoxin level in sesame seed sample

According to the finding described above in Table 4, 89%, out of 27 sesame samples, are positive for total aflatoxins with average of total aflatoxin content of 26.22 ng/g, 55% of the samples are positive for aflatoxin B1 in the range of 1.32-2.12 ng/g, 55% of the total samples are positive for aflatoxin B2 in the range of 1.52-31.98 ng/g, 55% of the sesame samples are positive for aflatoxin G1 in the range of 16.2-48.28 ng/g and 63% of the samples are positive for aflatoxin G2 within the range of 0.56-9.04 ng/g. As compared with aflatoxin contamination of groundnuts, sesame seeds are less contaminated, but still above the European Union limit for both individual and total aflatoxins.

Isolation and identification of aflatoxigenic fungi
Isolates of *Aspergillus flavus, Aspergillus niger* and *Aspergillus parasiticus* were identified from the sesame seeds cultured on PDA. *Aspergillus flavus* was isolated from the majority of the sesame seed samples cultured. Figure 3 depicts the fungal isolates obtained after culturing the sesame seed sample on PDA.

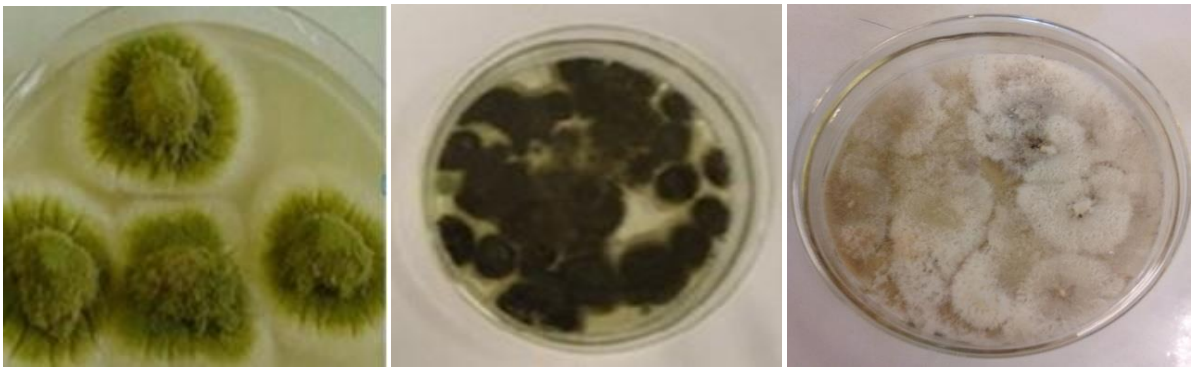

Fig. 3. Isolates of *A. flavus, A. niger and A. parasiticus* (from left to right)

Conclusion

In this study aflatoxin concentrations and the presence of Aspergillus fungi were determined in Ethiopian white and red export standard sesame seeds. According to knowledge, attitude and practice survey and seed characteristics studies, aflatoxins contamination detected from the sesame samples is highly associated with improper post-harvest handling of sesame seed. The extent of aflatoxins contamination of Ethiopian export standard sesame is low, as compared with aflatoxins level detected in sesame seed from Nigeria, Greece and China. Furthermore, the contamination level of the most of the sesame seeds was above the tolerable limit. Thus, this might interrupt Ethiopian sesame export market and affect country's economy by decreasing the revenue from sesame export. Therefore, stakeholders involved in sesame value chain from farm to market should work in collaboration to reduce aflatoxin contamination.

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