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GROWTH AND PRODUCTION OF AFLATOXINS BY *A. FLAVUS* IN AQUEOUS FRUIT EXTRACTS OF PEPPER, OKRA AND TOMATO

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ABSTRACT
The growth of *A. flavus* and its potential to produce aflatoxins in aqueous fruit extracts of pepper, tomato and okra were studied using conventional mycological techniques, and high performance liquid chromatographic analysis. At the end of the incubation period the maximum dry weight of 225±2.89mg was observed in tomato extract, whilst the lowest growth (73.3±3.33mg) was achieved in okra extract. In all the samples analyzed, aflatoxins G1 and G2 were completely absent, however over 60% filtrates of the fruits extracts were contaminated with aflatoxin B1. The highest amount (105.34µg/kg) of aflatoxin B1 was recorded in amended pepper extract whilst the least (0.85µg/kg) of aflatoxin B2 was recorded in the amended tomatoes extract. Only the pure tomato extract was contaminated with aflatoxin B1, no aflatoxins were also detected in pure extracts of pepper and okra. The implications of these findings on the health of both humans and animals are discussed.

KEY WORDS: *A. flavus*, aflatoxins, mycelium dry-weight, fruit extracts.

INTRODUCTION

Over the past decades, fungi have received special attention due to their ability to produce mycotoxins which are secondary metabolites with well-known health effects on both humans and animals. These mycotoxins are known to be produced by several fungal species in the genera of *Alternaria, Aspergillus, Eurotium, Cladosporium and Penicillium*. Fruits of pepper, tomato and okra, like many other fruits could be contaminated with spores or conidia and mycelial fragments from the environment. These contaminations may occur at different stages of production such as during growth and ripening of fruits, during transportation and processing of semi-completed and end products. The presence of potential toxigenic species on fruits and other food materials does not necessarily mean that the food materials contain mycotoxins. A variety of environmental factors such as temperature, water activity and oxygen may determine the type and the extent to which the toxigenic fungal species produce mycotoxins (Samson and Reenen-Hoekstra, 1988).The mycotoxins most commonly associated with fruits, vegetables and their products are aflatoxins, patulin, ochratoxin A and *Alternaria* toxins (Barkai-Golan and Paster, 2008) which are produced by members of the genera *Aspergillus, Penicillium* and *Alternaria*. *Aspergillus flavus* is one of the toxigenic fungal species that affects food in the tropical regions as well as *A. parasiticus* and *A. nomius* (Egel *et al*., 1994; Atehnkeng *et al*., 2008).These moulds, have the ability to invade and colonize a variety of food substances and produce aflatoxins depending on the prevailing conditions of temperature, relative humidity and moisture levels of the food (Rosi *et al*., 2007). About ten species of the genus *Aspergillus* are known to synthesize aflatoxins (Peterson *et al*., 2001; Frisvad *et al*., 2005; Kenjo *et al*., 2007; Ehrlich *et al*., 2007), and the two most commonest ones are *Aspergillus flavus* and *Aspergillus parasiticus* (Gourama and Bullerman, 1995). Aflatoxins B1, B2, G1 and G2 are thermostable compounds (Marasas and Nelson, 1987) and occur worldwide on a large variety of foods and feeds. These aflatoxins are known to be carcinogenic, teratogenic, and mutagenic (Busby and Wogan, 1981). They bind to DNA and prevent transcription of genetic information resulting in various adverse effects on humans and other animals (Atlas, 1995). They are both acutely and chronically toxic to animals including man causing acute liver damage, liver cirrhosis, induction of tumours and teratogenic effects (Stoloff, 1977). Muhammad *et al*. (2004) found eight different fungi, viz *Aspergillus niger, Aspergillus ochraceous, Aspergillus flavus, Aspergillus fumigatus*, *Penicilium citrinum, Curvularia lunata* and *Sclerotium rolfsii* associated with rotten tomato fruits obtained from five different markets in Nigeria. *A. flavus* and *A. niger* had the highest rate of occurrence among the isolated fungi (Muhammad *et al*., 2004). Studies conducted by Mensah and Owusu (2012) identified a total of 18 fungal species belonging to 8 genera from surface sterilized and non sterilized fruits of pepper, tomato and okra from the markets in Accra metropolis. The study also showed that members of the genus *Aspergillus* were dominant with *A. flavus* being the most dominant fungal species occurring on all fruits. Ahene *et al*. (2011) and Ezekiel *et al*. (2013) also identified *A. flavus* as the most frequently isolated fungal species in spices and spice product. The conidia of *A. flavus* are known to occur in the air and in the soil and their ability to

produce large number of enzymes enable them to utilize a variety of substances as food source. They thrive on any organic substance with little moisture and also on products with moisture levels above 16% (Suberu, 2004). The dominance of a particular fungal species on food material or product may be due to the characteristics of the species and the properties of the product. It may also be due to heavy contamination from ecological microhabitats where the mould had developed.

A number of mycotoxins have been associated with genus *Aspergillus* in fruits and vegetables. The predominant ones being aflatoxins and ochratoxin A which are harmful to humans and animals (Barkai-Golan and Paster, 2008). Of the known aflatoxins, B1 (AFB1) remains the most prevalent in foods (Lee *et al*., 2004). AFB1 is also the most potent toxic metabolite capable of inducing hepato-carcinogenicity (Sweeney and Dobson, 1998), genotoxicity in reproductive and blood cells (Fapohunda *et al*., 2008; Ezekiel *et al*., 2011). Studies conducted by Tsuchiya *et al*., (2011) indicated red chili peppers from Chile were contaminated with aflatoxins B1 and G1.Clearly, the predominance of *A. flavus* on fruits of pepper, tomato and okra predisposes the produce to severe losses, and may have adverse effects on both humans and animals due to their ability to produce potent mycotoxins (Mensah and Owusu, 2012). The objective of this research was to study the growth and the production of aflatoxins of *A. flavus* in the aqueous fruits extract of pepper, tomato and okra.

MATERIALS & METHODS

Preparation of aqueous fruits extract

Fruits extract were prepared by blending 250g of tomato, 50g each of pepper and okra in 250ml, 250ml and 500ml of sterilized distilled water, respectively, using the National MX-J210PN blender. Each suspension was then filtered with clean absorbent cotton wool and next with filter paper using Compton vacuum pump. The filtrate was autoclaved at 121^{o}C, 1.1kg/cm^{3} for 15 minutes and used as the growth medium for *A. flavus.*

Preparation of potato dextrose broth (PDB)

Peeled 200g of Irish potato tubers were cut into pieces and boiled in 500ml of distilled water for 10 minutes. The extract was strained with muslin cloth and made up to 1000ml. Ten grams of dextrose was added and heated in a boiling water bath and autoclaved at 121^{o}C, 1.1kg/cm^{3} for 15 minutes. The PDB was used to amend the fruit extracts.

Growth of *A. flavus* in aqueous fruits extract

The ability of *A. flavus* to grow in aqueous fruits extract of pepper, tomato and okra, and amended fruits extracts were investigated. An aliquot of 30ml of the extract was delivered into each 250ml Erlenmeyer flask. There were seven treatments with 15 replicates per treatment. The flasks were sterilized and then inoculated with 6mm disc from five day old *A. flavus* culture and incubated at 30^{o}C for 10 days. Three flasks were withdrawn at two days intervals and the vegetative growth was assessed separately by the mycelial oven dry weight method.

Assessment of Growth

Three flasks were withdrawn at two day intervals and the vegetative growth was assessed separately by the mycelial oven dry weight method. A metal spatula was used to dislodge the mycelia stuck to the sides of the Erlenmeyer flask and harvesting was done by filtering the contents of the flask with a previously dried and weighed Whatman No.1 filter paper and dried at 70 ^{o}C for 24hours. The dried filter paper and the mycelium were then weighed after it had been cooled in a desiccator and the dry weight of the mycelium determined. The final pH of the filtrate was also determined using the glass electrode pH meter (TOA, HM 605).

Aflatoxin analysis

Twenty five grams of the filtrate was weighed into blender jar at the end of the incubation period. 5g of NaCl, 200ml of methanol were added and blended for 3minutes using high speed Waring blender and filtered immediately. Ten millilitres of the filtrate was transferred into 250ml beaker and 60ml of Phosphate Buffered Saline (PBS) was added and stirred. The mixture was transferred onto conditioned immunoaffinity column (IAC) at a flow rate of approximately 3ml/min. The column was washed with approximately 15ml of water, applied in little portions of approximately 5ml/min and dried by passing air through the immunoaffinity column by means of a syringe for 10s. The aflatoxins were eluted by applying 0.5ml methanol on the column and allowed to pass through by gravity and after 1minute another 0.75ml methanol was applied on the column and the elution solvent was collected into 5ml volumetric flask by pressing air through. The volumetric flask was topped with distilled water to the mark and well shaken. Based on the clarity of the solution, it was used directly for High Performance Liquid Chromatography (HPLC) analysis or passed through a disposable filter unit (0.45 µm) prior to HPLC injection. The HPLC system was made up of a Degasser, Waters 1525 Binary HPLC pump, Waters 2707 Autosampler, Waters 2475 Multi λ Fluorescence Detector and Breeze 2 software. The mobile phase of HPLC was made of water: acetonitrile: methanol solution (6 + 2 + 3 (v+v+v). The various parameters for the fluorescence detector (Waters 2475) were set as follows: excitation 360nm, emission 440nm, attenuation 1, and gain (%) value 1. The post column reagent was prepared by dissolving 25mg of Pyridine hydrobromide per bromide (PBPB) in 500ml distilled H_2O. The post column derivatization was carried out using PBPB, by mounting a mixing T- piece and reaction tubing and then operated using the following parameters: Flow rates :1.0ml/min (mobile phase) and 0.3ml/min (reagent). Calibration curves were prepared using the working calibration solutions. These solutions covered the range of 1 ng/g- 4ng/g for aflatoxins B_1 and G_1 and the range of 0.2ng/g- 0.8ng/g for B_2 and G_2. The calibration curves were drawn and checks for linearity of the plots were done using Breeze 2 software. In the case where the content of aflatoxins in the sample fell outside the calibration range, an appropriate calibration curve was prepared. Alternatively the injection solution for HPLC analysis can be diluted to aflatoxin content appropriate for the established calibration.

RESULTS

Growth of *A. flavus* in aqueous fruits extract

Growth of *A. flavus* was variably affected by the fruit extracts. The highest growth was observed in fruit extracts

of tomato, followed by growth in pepper and lastly in okra extracts whilst the lowest growth (73.3mg) was achieved in okra extract (Table 6). Peaks of 225.0 and 126.7mg were attained in 4 days in tomato and pepper extracts, respectively, whereas it was 95.0mg in 6 days in okra extract. Vegetative growth of *A. flavus* in the amended pepper and okra extract were higher as compared to the pure extracts of pepper and okra (Tables 4, 5, 6 and 7). Figure 1, shows the vegetative growth of *A. flavus* in amended and pure okra extracts, with much sporulation occurring in the amended extract. Conversely, in the amended tomato extract growth was lower as compared to

growth in the pure tomato extract (Tables 1 and 2). Growth in PDB was higher than growth in unammended and amended extracts of pepper and okra but never reached the growth recorded in tomato extract or the amended tomato extract. It is also remarkable to note that with the exception of the pure and amended okra extracts in which autolysis began after the 6th day of incubation, in the tomato and pepper extracts, autolysis began after four days of incubation. In all the extracts, the pH of the filtrates shifted from the acidic medium to the alkaline region and it was very basic in the tomato extract as compared to pepper and okra extracts at the end of the incubation period. Moreover, the degree of alkalinity increased in all the test media as incubation period also increased.

TABLE 1: Mean dry weight of *A. flavus* grown in tomato extract at 30^{o}C for 10 days.

Incubation	pH of Medium		Dry wt. of mycelium (mg)
Period (days)	Initial	Final	Mean ± Standard Error
2	4.10	4.30	216.7±3.33^{bc}
4	4.10	7.45	225.0±2.89^{c}
6	4.10	7.89	208.3±1.67^{ab}
8	4.10	7.92	203.3±3.33^{a}
10	4.10	7.97	200.0±2.89^{a}

By calculated Confidence Limit, Means bearing the same letter are not significantly different at 95% Level Probability.

TABLE 2: Mean dry weight of *A. flavus* grown in tomato extract amended with at PDB 30^{o}C for 10 days.

Incubation	pH of Medium		Dry wt. of mycelium (mg)
Period (days)	Initial	Final	Mean ± Standard Error
2	4.37	5.55	205.0±2.89^{d}
4	4.37	7.40	216.7±3.33^{e}
6	4.37	7.46	195.0±2.89^{c}
8	4.37	7.57	180.0±0.00^{b}
10	4.37	7.58	163.3±2.89^{a}

By calculated Confidence Limit, Means bearing the same letter are not significantly different at 95% Level Probability.

TABLE 3: Mean dry weight of A. *flavus* grown in PDB at 30^{o}C for 10 days.

Incubation	pH of Medium		Dry wt. of mycelium (mg)
Period (days)	Initial	Final	Mean ± Standard Error
2	5.61	5.68	126.7±3.33^{b}
4	5.61	6.42	168.3±1.67^{d}
6	5.61	6.56	138.3±1.67^{c}
8	5.61	6.75	123.3±3.33^{b}
10	5.61	6.81	113.3±3.33^{a}

By calculated Confidence Limit, Means bearing the same letter are not significantly different at 95% Level Probability.

TABLE 4: Mean dry weight of *A. flavus* grown in pepper extract at 30^{o}C for 10 days.

Incubation	pH of Medium		Dry wt. of mycelium (mg)
Period (days)	Initial	Final	Mean ± Standard Error
2	4.93	4.98	86.7±1.67^{a}
4	4.93	6.61	126.7±3.33^{d}
6	4.93	6.90	113.3±3.33^{c}
8	4.93	7.15	110.0±0.00^{bc}
10	4.93	7.42	103.3±3.33^{b}

By calculated Confidence Limit, Means bearing the same letter are not significantly different at 95% Level Probability.

TABLE 5: Mean dry weight of *A. flavus* grown in pepper extract at 30°C for 10 days.

Incubation	pH of Medium		Dry wt. of mycelium (mg)
Period (days)	Initial	Final	Mean ± Standard Error
2	5.09	4.80	113.3±3.33[a]
4	5.09	6.60	138.3±1.67[c]
6	5.09	6.65	126.7±3.33[b]
8	5.09	6.72	116.7±3.33[a]
10	5.09	6.92	110.0±0.00[a]

By calculated Confidence Limit, Means bearing the same letter are not significantly different at 95% Level Probability.

TABLE 6: Mean dry weight of *A. flavus* grown in okra extract at 30°C for 10 days.

Incubation	pH of Medium		Dry wt. of mycelium (mg)
Period (days)	Initial	Final	Mean ± Standard Error
2	6.00	5.70	73.3±3.33[a]
4	6.00	6.16	86.7±3.33[bc]
6	6.00	6.22	95.0±2.89[c]
8	6.00	6.26	85.0±2.89[b]
10	6.00	6.37	81.7±1.67[ab]

By calculated Confidence Limit, Means bearing the same letter are not significantly different at 95% Level Probability.

TABLE 7: Mean dry weight of *A. flavus* grown in okra extract amended with PDB at 30°C for 10days.

Incubation	pH of Medium		Dry wt. of mycelium (mg)
Period (days)	Initial	Final	Mean ± Standard Error
2	5.71	5.43	93.3±3.33[ab]
4	5.71	6.15	100.0±0.00[b]
6	5.71	6.38	113.3±3.33[c]
8	5.71	6.45	98.3±1.67[b]
10	5.71	6.60	90.0±0.00[a]

By calculated Confidence Limit, Means bearing the same letter are not significantly different at 95% Level Probability

FIGURE 1: Photograph showing the growth of *Aspergillus flavus* in aqueous okra extract incubated at 30°C after 4 days of incubation. (x3/7) left: Unamended extract. Right: Amended extract (note the degree of sporulation).

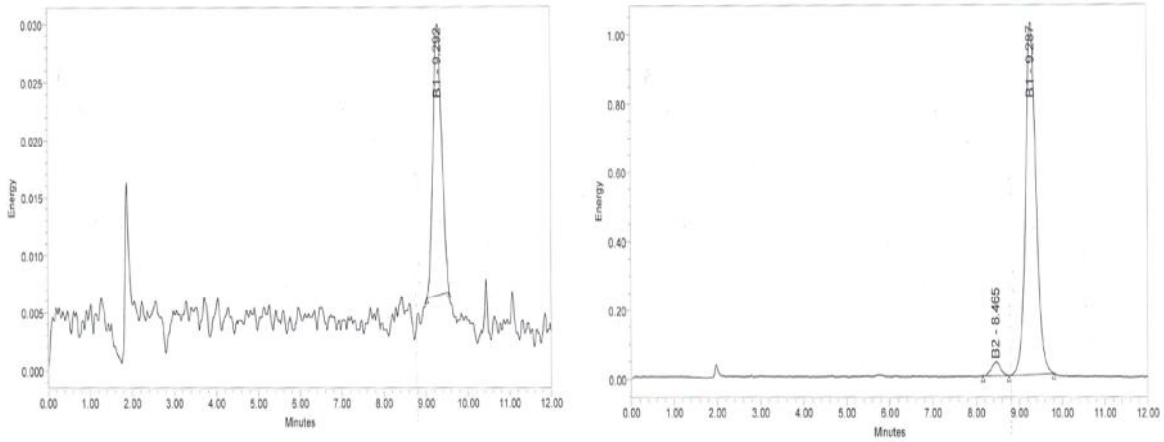

FIGURE 2: The incidence of aflatoxin in tomato extract (left) and amended tomato extract (right)

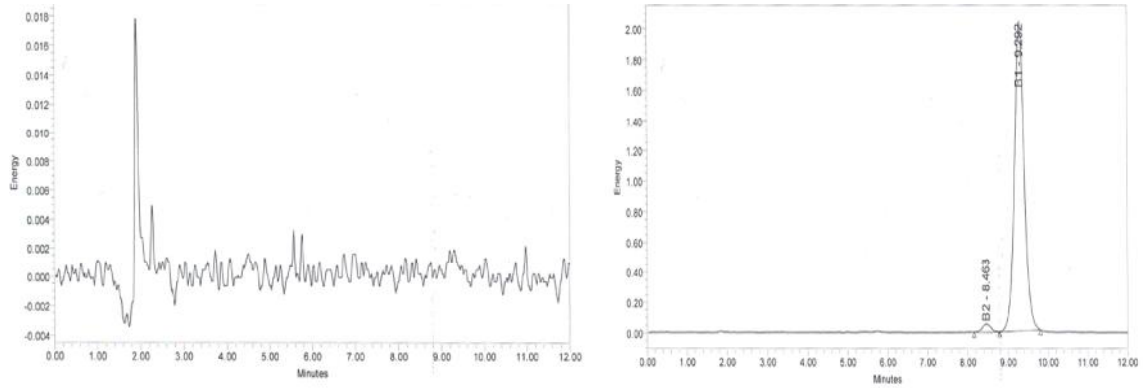

FIGURE 3: The incidence of aflatoxin in pepper extract (left) and amended pepper extract (right).

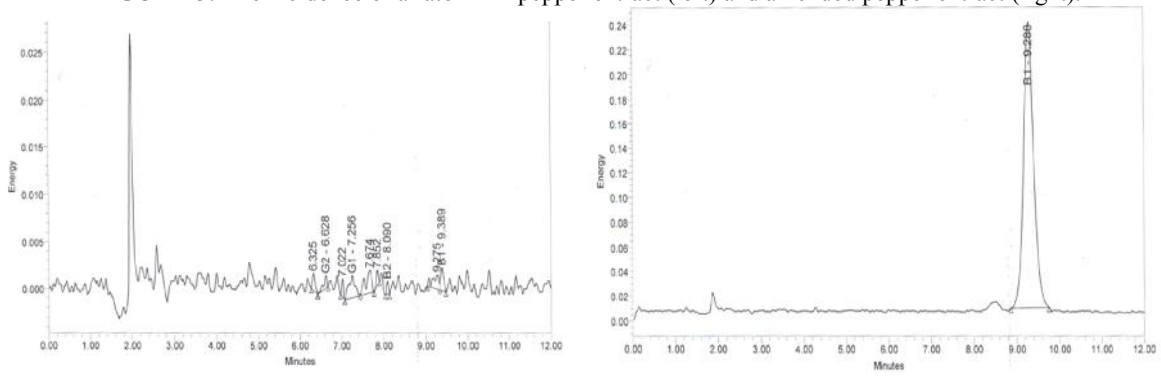

FIGURE 4: The occurrence of aflatoxin in okra extract (left) and amended okra extract (right)

TABLE 8: Occurrence of aflatoxins in aqueous fruits extract of pepper, tomato and okra.

Treatment/ Sample	Aflatoxins (µg/kg)				Total Aflatoxins (µg/kg)
	G2	G1	B2	B1	
TTP	ND	ND	ND	1.04	1.04
TTA	ND	ND	0.85	52.82	53.67
PPP	ND	ND	ND	ND	ND
PPA	ND	ND	1.20	105.34	106.54
DKP	ND	ND	ND	ND	ND
DKA	ND	ND	ND	11.75	11.75

ND-Not Detected, TTP-tomato extract, TTA-amended tomato extract, PPP-pepper extract, PPA- amended pepper extract, DKP-okra extract, DKA-amended okra extract.

Aflatoxins analysis

The occurrence of Aflatoxins in the aqueous fruits extract of pepper, tomato and okra were assessed at the end of the incubation period. Over 60% of the test samples were contaminated with aflatoxin B1 and B2. However, aflatoxins G1 and G2 were completely absent in all the test samples. The absence of these mycotoxins could be that, their concentrations were probably below detection limits or were completely not formed by the test fungus. The highest amount of aflatoxin B1 (105.34µg/kg) was recorded in the amended pepper extract (PPA) whilst the least (0.85µg/kg) of aflatoxin B2 occurred in the amended tomato extract (TTA) samples. Interestingly with all the pure extracts, only the pure tomato extract was contaminated with aflatoxin B1. No aflatoxins were detected in pure samples of pepper (PPP) and okra extracts (DKP) as indicated in Table 8.

DISCUSSION

The growth of *A. flavus* differed in the fruits extract and the amended extracts. This is not surprising, because reports in pertinent literature, clearly indicates differences in growth of some fungi on agar and in liquid medium (Carlile *et al*. 2005). Fungi are selective in their ability to utilize sugars and other nutrients and that nutritional requirements differ among species and sometimes even among strains of the same species. Again not only the type but also the concentration of the carbon source is important in determining how effective it can promote growth of fungi (Garraway and Evans, 1984).

The differences observed in the vegetative growth in all instances may be partly attributed to the variation in nutrient status of the growth medium used for the test fungi. According to Norman, (1992), variations exist in the nutritional status of fruits of pepper, okra and tomatoes.

The optimum pH for growth of fungi varies considerably with the species and the medium used (Garraway and Evans, 1984). In all instances, the pH of the growth medium shifted from acidic to basic side (Tables 1-7). *A. flavus* has the ability to produce a number of enzymes which enables the fungus to develop on a wide variety of stored grains such as wheat, peanuts, soybeans, corn, groundnut and oil foods and produce toxins (CDC, 2004). The production of toxins by *A. flavus* has been demonstrated on several food products and under optimal conditions of growth, some toxins can be detected within 24hours, or generally within 4-10 days (Christensen, 1971).

Although *A. flavus* was the most constantly isolated fungal species on spices and spice products, no aflatoxins were detected in the extracts of the spices (Ahene *et al*., 2011). None of the fruits extracts analyzed in this study was contaminated with aflatoxins G1 and G2, which may be attributed to low concentrations below detection limits. According to Marasas and Nelson, (1987) aflatoxins B1, B2, G1 and G2 occur on a large variety of foods and feeds due to their thermostability in nature. Of the known aflatoxins, aflatoxin B1 (AFB1) remains the most prevalent in foods (Lee *et al*., 2004) and the present study also affirms the dominance of AFB1 in the aqueous fruits extracts.

The natural incidence of aflatoxins in foods and feeds is influenced by climate and practically all tropical countries encounter the problem of mycotoxicoses (Cardona *et al*. 1991). AFB1 has been shown to be the most potent toxic metabolite capable of inducing hepato-carcinogenicity genotoxicity in reproductive and blood cells (Fapohunda *et al*., 2008; Ezekiel *et al*., 2011). Aflatoxins are known to be immunosuppressive, mutagenic, teratogenic and carcinogenic in their effect with the main target organ being the liver. It causes acute liver damage, liver cirrhosis, induction of tumours and teratogenic effects (Stoloff, 1977). There have been a number of reported cases of aflatoxicosis in Africa associated with the consumption of contaminated maize. In Kenya, 12 and 125 people died in 1982 and 2004 respectively (Ngindu *et al*., 1992; CDC, 2004) due to aflatoxicosis. Studies conducted by Samyal and Sumbali (2012) on diseased tomato fruits of cv. Marglobe showed a considerably high amounts of AFB1 and AFB2 which were beyond the regulatory limit of 20 µg/kg fixed by the World Health Organization (WHO). Tsuchiya *et al*., (2011) indicated red chili peppers were contaminated with aflatoxins B1 and G1. A survey of aflatoxin contamination of rotten tomatoes from 5 local markets in Nigeria also showed aflatoxin contamination even after autoclaving at 121 °C for 15 min (Muhammad *et al*., 2004). The current study has also shown the production of aflatoxins in the pure and amended aqueous fruits extracts of pepper, tomato and okra. Although pure extracts of tomato was contaminated with AFB1, the level (1.04µg/kg) was far below the permissible safe limits for human consumption and health. Research conducted in some West African countries also reveals that dried samples of okra and hot chilli were contaminated with AFB1 and AFB2 (Hell *et al*., 2009).

It is therefore essential that future studies on stored fruits for sale on markets be analyzed for the presence of mycotoxins especially; aflatoxins since the present study has demonstrated the presence of aflatoxins in aqueous fruits extracts of pepper, tomato and okra.

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