

Identification of Aflatoxigenic Fungi and Detection of Their Aflatoxin in Red Chilli (*Capsicum annuum*) Samples Using Direct Cultural Method and HPLC

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Abstract

Aflatoxins are the potential lethal toxin produced by *Aspergillus* sp. important health hazard throughout the world. In this study, 26 *Aspergillus* sp. have been isolated from 50 samples of red chilli collected throughout the country. These 26 isolates were grown primarily on agar media to identify the aflatoxin producing species. It is possible to distinguish *A. flavus* strains from other *Aspergillus* sp. developing orange colour on the reverse of the plates. The Coconut Cream Agar (CCA) is used to detect aflatoxin producer strains having blue fluorescence when exposed to a UV-light. Several other media were used for morphological characteristics of *Aspergillus* sp. Out of 26 isolates, four isolates were confirmed as *Aspergillus* sp. These isolates were subjected to cross contamination with freshly ground, sterile maize and after 15 days of incubation the contaminated maize were analyzed by HPLC and found aflatoxin in each of the sample containing 186 ppb (max.). This study was conducted to assay the ability to produce aflatoxins by the *Aspergillus* spp. isolated from red chilli (*Capsicum annuum* L. Solanaceae) available throughout the country. The results found in the experiment are much more behind the acceptable limit according to some international standard. As red chilli is a widely used spice in Bangladesh, the proper controlling measures may be taken for controlling the surveillance of aflatoxinic fungi like as use of bio-pesticides, proper drying method and storage conditions.

Keywords

Aflatoxin, *Aspergillus*, Detection, HPLC, Red Chilli

1. Introduction

Chilli (*Capsicum annum* L. Solanaceae) is one of the most valuable spices and also important cash crops grown in Bangladesh. It is available in the form of green, dried and powdered. It has become an essential ingredient in Bangladeshi meals as it is typically lightly fried with oil in the initial stages of preparation of the dish [1]. According to the survey of Bangladesh Bureau of Statistics [2], plenty of chillies are being produced in the district of Bogra, Rangpur, Kurigram, Jamalpur, Natore and Jessore. It is an important spice in Bangladesh for its wide uses in cooking as well as nutritional value. It has become an essential ingredient in Bangladeshi meals. Every day billions of people use it as a medicinal herb and ornamental plant [3]. It is also used as an ingredient in industrial products. Chilli is an important source of vitamins such as A, C and E for the world population. They are powerful antioxidant and anti-inflammatory agents containing high amounts of carotenoid. The ascorbic acid (vitamin C) and tocopherol (vitamin E) content in chilli is quite high, which makes it very effective as immune system stimulants and healing agent especially for cellular damage [4]. But a high level of consumption of red chili was demonstrated to be an environmental risk factor as it is highly susceptible to fungal contamination and subsequently mycotoxin formation. Chilli is recognized as significant carrier of microbial contamination, primarily molds and some bacteria [5]. Chillies have been reported as one of the crops with high aflatoxin contamination. Temperature and humidity are the crucial factors that can influence the toxin production before and after harvest of the crop [6]. Delayed harvest, late irrigation, rain and dew during warm periods are associated with increased aflatoxin levels [7]. Improper picking practices and offensive post-harvest processing of the chillies may lead to augmented aflatoxin production. Aflatoxin contamination may also take place due to the mechanical damages, stress conditions or damages by birds, mammal or insects etc. [8].

Aflatoxins are the secondary metabolites and potent carcinogens produced by various species of *Aspergillus* [9]. Aflatoxins are the most potent carcinogenic toxins and widely studied of all mycotoxins. Aflatoxins frequently contaminate agricultural commodities so that causing serious health hazards to humans and animals, as well as great economic loss. Although aflatoxin-production ability has been detected in various species of the *Aspergillus* genus, inside and outside the Flavi group, *Aspergillus flavus* and *Aspergillus parasiticus* remain the most important and representative aflatoxin producers occurring naturally in food commodities [10]. The identification of *Aspergillus* Section Flavi has been based on morphological and biochemical characterization. The primary morphological

diagnostic character for separation of *A. flavus* and *A. parasiticus* is conidial wall ornamentation. When isolates are grown on Czapek-Dox (CZ) media, colonies of *A. flavus* are yellow-green and those of *A. parasiticus* have a distinctly darker green [11] [12].

Several types of aflatoxins are produced in nature belonging to a group called the difuranocoumarins, only four, aflatoxin B1 (AFB1), aflatoxin B2 (AFB2), aflatoxin G1 (AFG1), and aflatoxin G2 (AFG2). These toxins are highly carcinogenic and elicit a wide spectrum of toxic effects when foods and feeds contaminated with aflatoxins are ingested. Aflatoxin may alleviate the risk of different diseases including liver cancer, cirrhosis, gastritis etc. Furthermore, the International Agency for Research on Cancer (IARC) acknowledges that there is sufficient evidence in humans for the carcinogenicity of naturally occurring AFB1 and mixtures of aflatoxins [13] with a role in the etiology of liver cancer. AFB1 is the most potent of all aflatoxins known to date and is generally found in the highest concentration in food and animal feeds [14]. As per the recently introduced European Union (EU) regulations, only 5 µg per kg of aflatoxin B1 and 10 µg per kg of total aflatoxins are allowed in chilli [15].

Aflatoxin biosynthesis pathway involves at least 23 enzymatic reactions and almost 15 structurally well-defined aflatoxin intermediates have been identified in aflatoxin biosynthesis 4 - 6. Totally 25 identified genes involved in aflatoxin biosynthesis are clustered within a 70-kb DNA region in the chromosome [16]. However, nowadays polymerase chain reaction (PCR), have been developed for testing the presence of pathogens, as well as for confirmation and genotyping of isolates from samples. The biosynthetic pathway for aflatoxin production by *A. flavus* has been elucidated, and genes in the aflatoxin biosynthetic pathway have been identified [17]. The *AflR* gene plays an important role in the aflatoxin biosynthetic pathway by regulating the activity of other structural genes such as *omt-A*, *ver-1*, and *nor-1*. More recently, aflatoxin production and aflatoxigenic strains differentiation are being assessed by monitoring the expression of aflatoxin genes using the reverse transcription polymerase chain reaction (RT-PCR) and real-time PCR methodologies. Such systems have been applied to monitor aflatoxin production and aflatoxin gene expression based on various regulatory and structural aflatoxin pathway genes in *Aspergillus parasiticus* and/or *Aspergillus flavus* and were found to be very rapid and sensitive [18].

Conventional methods used for the detection of aflatoxins are microbiological identification, high performance liquid chromatography (HPLC), thin layer chromatography (TLC) or enzyme-linked immune sorbent assay (ELISA) [19]. New instrumental techniques approaches for aflatoxin determination such as fluorescence polarisation, multiphoton-excited fluorescence, LC separation, electrospray ionisation-MS-MS, liquid chromatography-electrospray ionization/multi-stage mass spectrometry (LC/ESI-MS-MS) detection [20] were developed but all these alternative methods are not always available or affordable to developing countries [21]. High-performance liquid chromatography (HPLC) of ex-

tracts of commodities, foods and feeds is the most prevalent and sensitive current method for the identification and quantization of mycotoxins. The detection of concentration of the fluorescent mycotoxins AFB₁, AFB₂, AFG₁, AFG₂ can be achieved by careful preparation of extracts of grain/fruit samples by HPLC [22]. The study was designed to isolate and identify aflatoxigenic fungi and aflatoxin from red chilli sample throughout the country Direct Visual Cultural Method and using high-performance liquid chromatography (HPLC).

2. Materials and Methods

2.1. Collection of Samples

A total of 50 chilli samples (250 gm each) were collected from different districts of Bangladesh for this study. Samples were brought to the laboratory and stored at 4 °C until being used [23].

2.2. Isolation and Screening of Aflatoxicogenic Fungi from the Chilli Samples

25 gm of chillies from each sample were dissolved in 225 ml of ringer solution separately and each sample was serially diluted up to 10⁻⁶ by vortexing. *Aspergillus* differentiation agar (AFPA) medium which is a selective identification medium for the isolation of *Aspergillus* group strains [24] was then inoculated with 0.1 ml of each dilution for the screening of strains of *Aspergillus* which are the dominant aflatoxigenic fungi by using spread plate technique incubating at 25 °C for 7 days. Development of a yellowish orange color of the colonies at the reverse side of the plate indicates the presence of *Aspergillus flavus* whereas green colored colonies indicate the presence of *Aspergillus parasiticus* [25]. All isolates were maintained in 20% glycerol at -20 °C and grown on Malt Extract Agar (MEA: Malt 20 g/L, Glucose 20 g/L, Peptone 1 g/L, Agar 20 g/L) in the dark for 7 days at 25 °C whenever needed for further studies [26].

2.3. Aflatoxin Producing Isolates in Different Culture Media and Detection with Fluorescence

Different media were used for growing of aflatoxigenic *Aspergillus* spp. These include Czapek dox agar medium, Coconut Cream agar (CCA) medium, Malt extract agar (MEA) medium, Rose Bengal Chloramphenicol agar medium and Potato Dextrose Agar (PDA) medium. However, the toxin production in these media varies with extraneous factors such as pH, temperature and time. Incubation for a period of five days is necessary for toxin production. All the isolates were kept at 25 °C for 5 - 7 days. In case of culturing with Coconut Cream agar (CCA) medium, the plates were incubated at 25 °C for 4 days in the dark, and the presence or absence of fluorescence on the agar surrounding the growing *Aspergillus* colonies was determined by exposing the Petri dishes to ultraviolet (UV) light (365 nm) and expressed as positive or negative [27].

2.4. Detection of Aflatoxin in Wheat and Maize

The aflatoxin producing ability of the isolates was tested by inoculating isolated strain in uninfected wheat and maize as these are mostly consumed cereal grains in Bangladesh. First the uninfected maize and wheat were disinfected by autoclaving in a flask and then manually inoculated with isolated *Aspergillus* spp. strains. Then the conical flasks were kept at room temperature for 15 days. The infected wheat and maize were carried out for the detection of aflatoxin by HPLC.

2.5. Determination of Aflatoxin Level by High-Performance Liquid Chromatography (HPLC)

2.5.1. Sample Preparation

Extraction was carried out according to extraction method taking 50 gram sample by making slurry with water at 1:2 ratio of sample: water. An appropriate volume of acetone was added to produce acetone to water ratio 1:4 and shaken in a mechanical shaker for 30 minutes and then collected the filtrate through a Whatman no. 1 filter paper in a conical flask. 10 ml methanol and 1 ml lead acetate was added to 10 ml of the filtrate in a 250 ml measuring cylinder and was made up to 150 ml with distilled water [28].

2.5.2. Clean up

Clean up was done using SPE Cartridge-3 ml, 500 mg PH packing was attached to 75 ml reservoir and a vacuum manifold. The cartridge was conditioned by passing of 15 ml methanol followed by 15 ml water under vacuum after adding 1 gram methanol washed celite. Then 150 ml prepared sample solution was passed through cartridge under vacuum at the rate of 10 ml/ min. The cartridge was then washed with 10 ml of water. Any remaining water from the cartridge was removed by the passage of air for about 5 minutes. The 75 ml reservoir was replaced with a 25 ml reservoir and successively another reservoir (4 ml) containing anhydrous sodium sulphate (500 mg) and inserted between the cartridge and vacuum manifold. The aflatoxins were eluted using 4 ml of chloroform at the rate of 0.5 ml/min in a 7 ml vial. The vial was dried under the stream of nitrogen at 45°C in a sample concentrator and reconstituted with 1 ml methanol and water (1:1) for HPLC analysis.

2.5.3. Sample Analysis

Samples were analyzed using HPLC system-Agilent: Liquid chromatography consist of Agilent: Solvent delivery system (pumps) Series 1100, Agilent series 1100 Column oven Agilent 1200 series Fluorescence detector, Manual injector and Cobra cell for post column derivatization. Software: Agilent ChemStation. HPLC Column was C18, 250 mm (L) × 4.6 mm (ID) 10 µL (Grace). Mobile phase was 630 ml water, 220 ml methanol, 150 ml acetonitrile, 120 µL of concentrated Nitric acid and 100 mg potassium bromide in isocratic mode with 1 ml flow rate. Total run time was 15 Min and Injection volume was 20 µL. Colum

oven temperature was 30°C and excitation wavelength 365 nm, emission wavelength 464 nm. Recovery was calculated for aflatoxins (B1, B2, G1 and G2) fortified at 2 ug/kg, 10 ug/kg, 20 ug/kg, 100 ug/kg and 200 ug/kg levels using peak area of chromatograms at concentrations ratio 5:1 (B1, G1: B2, G2) of standards and was found 87% - 92%. Suitable seven point calibration curve was done, preferably on matrix at 0.5, 2, 10, 25, 50, 100 and 250 ng/ml (ug/kg) level. Linear regression was 0.99. The calibration batch was prepared from the mixed working standard of 1000 ug/kg in methanol and water (1:1) which was prepared from stock standard of 20 ppm in acetonitrile. A control spiked samples of 2 ug/kg, 50 ug/kg and 200 ug/kg was run after every 10 samples followed by a solvent as blank. The method was validated as per European commission decision (Commission decision, 2002). The limit of detection was 0.5 ug/kg, Decision Limit ($CC\alpha$) was 4.34 ug/kg and Detection Capability ($CC\beta$) was 4.64 ug/kg.

3. Results and Discussion

3.1. Morphological Identification of Aflatoxin Producing *Aspergillus*

A total number of 26 colonies were got from 50 chilli samples. By culturing the samples in AFPA media (**Figure 1(a)**), it is possible to distinguish these species from other *Aspergillus* based on the development of orange color on the reverse of the plates. Four potential isolates were selected in this study. The CCA is used to detect aflatoxin producer strains (**Figure 1(b)**). When exposed to a UV-light, the production of aflatoxin is detected by a green-blue fluorescence [29]. When grown on CZ, colonies between the two species can also be separated taxonomically (**Figure 2(a)** & **Figure 2(b)**). The yellow-green color producing colonies are regarded as *A. flavus* and darker green color producing colonies are considered as *A. parasiticus* [30]. The aflatoxin-producing isolates were identified by cultivating fungal strains in Czapek dox agar medium for 5 days at 25°C [31].

For growing of aflatoxigenic *Aspergillus* sp. different media were used. The colony morphology of the isolates is summarized at **Table 1**.

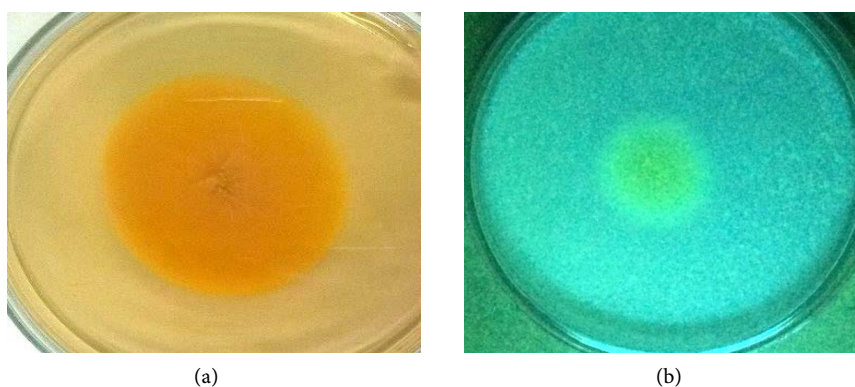


Figure 1. (a) *A. flavus* in AFPA, after 7 days incubation at 25°C, with the characteristic orange color on the reverse side of the plate; (b) Aflatoxigenic *A. flavus* grown on plates of CCA under long-wave UV light, after 7 days incubation.

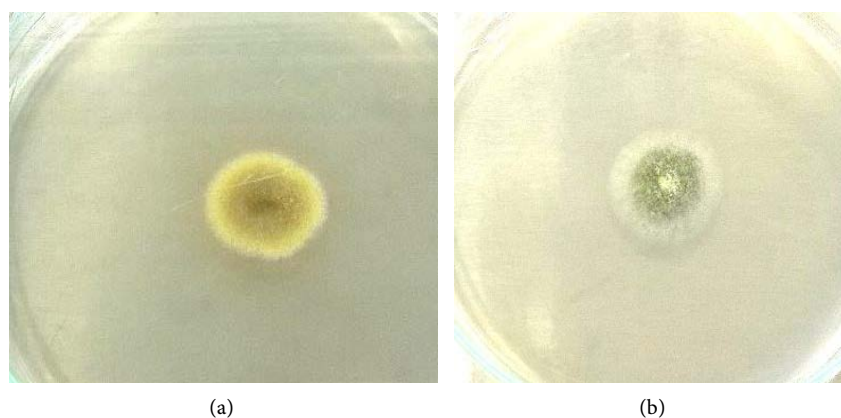


Figure 2. *A. flavus* (a) and *A. parasiticus* (b) strains growing on CZ media.

Table 1. Morphological characteristics of aflatoxigenic *Aspergillus* in different media.

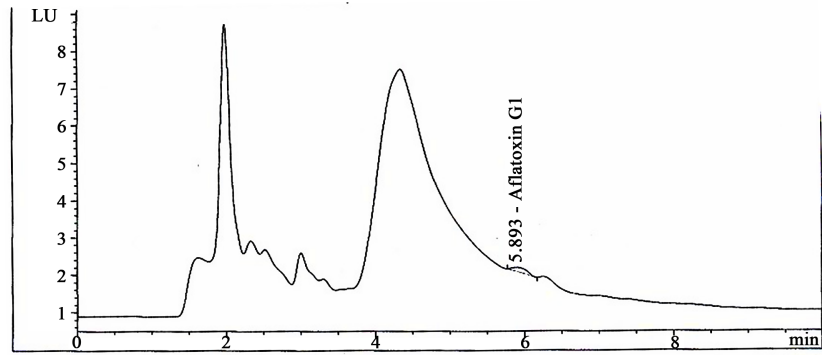
Name of Media	Colony Morphology
Malt Extract Agar (MEA)	Dark green to yellow colony with white edge.
Rose Bengal Chloramphenicol Agar Medium	Yellow to green colony with white edge.
Sabouraud Dextrose Agar (SDA)	Whitish colony.
Potato Dextrose Agar (PDA)	Lime green to yellow colony with white edge
Yeast Extract Agar (YEA)	Dark green to yellow colony with white edge.

Different types of media were being used for growth and culture preservation but Malt Extract Media (MEM) gave the best possible growth of *A. flavus* strains [32], [33] and [34]. The Rose Bengal chloramphenicol agar medium has been used to isolate *A. flavus* group fungi numerous samples such as nuts, peanuts, corn, cereal, spices and diverse soil samples. This was reported by [35] demonstrated that Sabouraud Dextrose and Yeast Extract agar media was used efficiently to detect aflatoxin producing *Aspergillus flavus* after 3 days of incubation at 28°C. He also evaluated that the using of those media at fifth or sixth day of incubation became difficult because mycelial growth reached almost the margin of the Petri dish. Potato dextrose agar is a suitable media for growth of aflatoxin producing *Aspergillus flavus* [36].

3.2. Detection of Aflatoxin by HPLC

Based on the visual culture method and morphological studies, twenty six *Aspergillus* strains were isolated and purified. The results of visual culture method were confirmed by high performance liquid chromatography (HPLC) method and analysis of aflatoxin production in red chilli samples were shown (Figure 3(a) & Figure 3(b)) [32].

As the sample collected directly analyzed by HPLC, found max. 68.674 ppb and 12.052 ppb min. Almost 75% of collected raw sample contain Aflatoxin (G2, G1, B2, B1). All isolates which showed pink/red colour at their reverse had strong signal of AFB1 in their HPLC chromatogram.



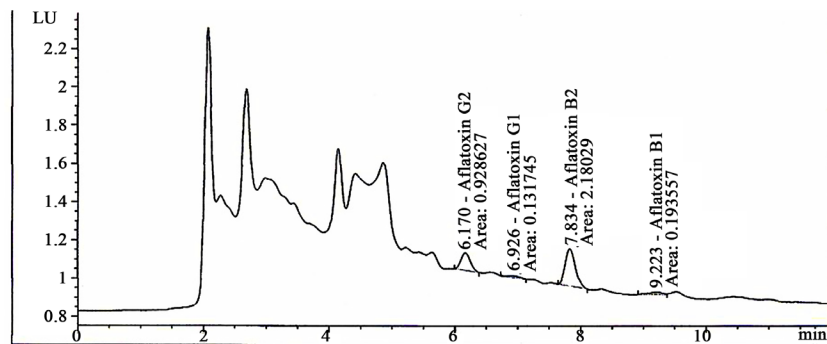
External Standard Report

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 Use Multiplier & Dilution Factor with ISTDs

Signal 1: FLD1 A, Ex = 365, Em = 418

Ret time [min]	Type	Area LU*s	Amt/Area	Amount [ppb]	Grp	Name
5.100						Aflatoxin G2
5.893	VP	1.61843	42.43259	68.67431		Aflatoxin G1
6.630						Aflatoxin B2
7.950						Aflatoxin B1
Totals:				68.67431		

(a)



External Standard Report

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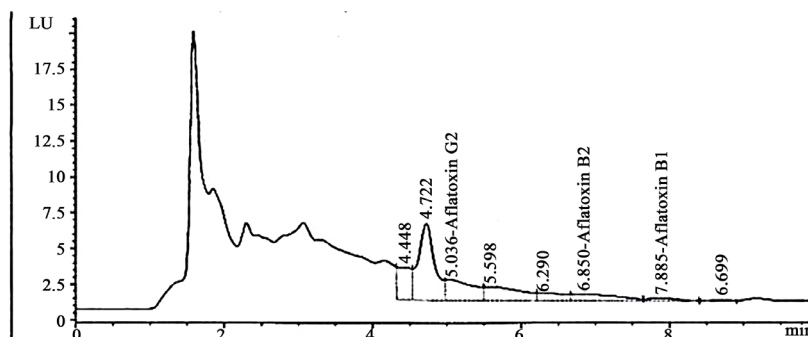
Signal 1: FLD1 A, Ex = 365, Em = 418

Ret time [min]	Type	Area LU*s	Amt/Area	Amount [ppb]	Grp	Name
6.170	MM	9.28627e-1	3.39799	3.15546		Aflatoxin G2
6.926	MM	1.31745e-1	42.34976	5.57936		Aflatoxin G1
7.834	MM	2.18029	3.56099e-1	7.76398e-1		Aflatoxin B2
9.223	MM	1.93557e-1	13.13123	2.54165		Aflatoxin B1
Totals:				12.05287		

(b)

Figure 3. (a) & (b) HPLC chromatogram of aflatoxin from red chilli sample.

The cross contaminated samples were analyzed and it is remarkable that max. 186.518 ppb where AFB1 55.693 ppb and 26.497 ppb min where AFB1 10.226 ppb aflatoxin was found (**Figure 4(a)** & **Figure 4(b)**) [37]. It is noted that the maximum acceptable limit for AFB1 is 2 ppb and 4ppb for overall aflatoxin



External Standard Report

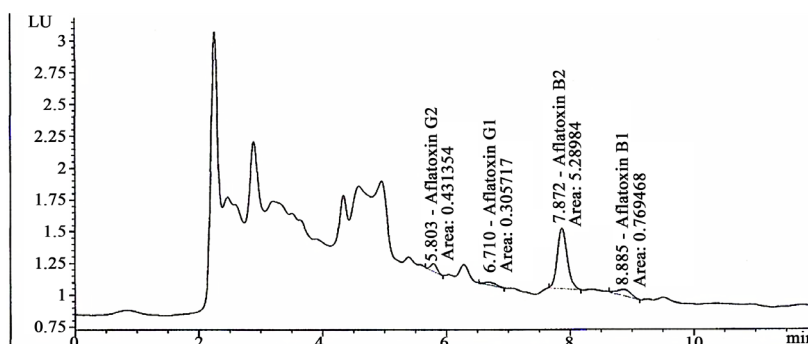
Sorted By : Signal
 Calib. Data Modified : 7/6/2015 1:26:29 PM
 Multiplier : 1.0000
 Dilution : 1.0000
 Use Multiplier & Dilution Factor with ISTDs

Signal 1: FLD1 A, Ex = 365, Em = 418

Ret time [min]	Type	Area LU	Amt/Area *s	Amount [ppb]	Grp	Name
5.036	VV	36.56425	3.40781	124.60388		Aflatoxin G2
5.970	VV	17.98864	3.45834e-1	6.22109		Aflatoxin G1
6.850	VV	4.17685	13.33376	55.69315		Aflatoxin B2
7.885	VP					Aflatoxin B1

Totals: 186.51812

(a)



External Standard Report

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 Dilution : 1.0000
 Use Multiplier & Dilution Factor with ISTDs

Signal 1: FLD1 A, Ex = 365, Em = 418

Ret time [min]	Type	Area LU	Amt/Area *s	Amount [ppb]	Grp	Name
5.803	MM	4.31354e-1	3.38637	1.46072		Aflatoxin G2
6.710	MM	3.05717e-1	42.40107	12.96272		Aflatoxin G1
7.872	MM	5.28984	3.49233e-1	1.84739		Aflatoxin B2
8.885	MM	7.69468e-1	13.29018	10.22636		Aflatoxin B1

Totals: 26.49720

(b)

Figure 4. (a) & (b) HPLC chromatogram of aflatoxin from cross contaminated sample.

according to European Union standard. In this study, it was observed that most of the dry red chilli available in Bangladesh contaminated with *Aspergillus* sp. containing a considerable amount of aflatoxin, which is a lethal mycotoxin. Necessary steps should be taken to control the spread-out of aflatoxin producing *Aspergillus*.

4. Conclusion

This study was conducted to assay the ability to produce aflatoxins by the *Aspergillus* spp. isolated from red chilli (*Capsicum annuum* L. Solanaceae) available throughout the country. The results found in the experiment are much more behind the acceptable limit according to some international standard. As red chilli is a widely used spice in Bangladesh for food preparations, contamination of red chilies has both health and economic implications. Thus extensive research need to be done to identify the exact factors responsible for aflatoxin contamination in red chili and proper controlling measures may be taken for controlling the surveillance of aflatoxinic fungi like as use of bio-pesticides, proper drying method and storage conditions. This type of practice may help the chili growers to maintain the required standards quality of their produce and be capable to supply aflatoxin free chili to the processors. Consequently, the availability of safe chili for domestic population and export will be a step forward to combat the food safety issues and to support the economic affairs of Bangladesh.

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