FUNGI ISOLATION, IDENTIFICATION AND DETECTION OF AFLATOXINS IN SELECTED CEREALS AND CEREAL PRODUCTS

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ABSTRACT

Background: The study aimed to detect toxigenic fungi and aflatoxin residues (B1, B2, G1, G2) in cereals and cereal products samples such as oats, rice, corn, corn flakes, wheat flour, and rice flour from local markets near Dhaka University, Bangladesh, due to the serious health risks posed by aflatoxins, produced by fungi *Aspergillus flavus*.
Methods: A total of 130 samples were collected and the pooled 26 samples were analyzed. Associated fungi were isolated and cultured on a PDA medium and identified morphologically based on colony color, texture, diameter, and microscopic observation. Molecular identification was done by sequence analysis of the Internal Transcribed Spacer (ITS) region, confirming the presence of *Aspergillus flavus*. Aflatoxin residues were detected using High-Performance Liquid Chromatography (HPLC) with a fluorescence detector and the QuEChERS extraction method.
Results: Out of 26 analyzed samples, 73.03% tested positive for aflatoxin residues (B1, B2, G1, G2), with an average concentration of 2.17 µg/kg. Two samples (wheat flour sample 3 and corn flakes sample 2) exceeded the Maximum Residue Limits (MRL) for aflatoxin B1 (2 µg/kg) and total aflatoxins (4 µg/kg) set by Commission Regulation (EC), representing 10.52% of the samples. *Aspergillus* spp., the primary producer of aflatoxins, was identified in 92.31% of the samples, alongside nine fungal genera and 19 fungal species.
Conclusion: The study emphasizes the health risks of aflatoxin residues in foods like corn flakes and oats in Bangladesh, which may probably associated with serious conditions like cirrhosis and hepatocellular carcinoma, urging prompt action for food safety.

KEYWORDS: Fungi, Aflatoxin, HPLC, QuEChERS, MRL, EC.

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Introduction

Cereal grains and their products are fundamental to human nutrition and animal feed, providing essential nutrients such as carbohydrates, proteins, fatty acids, and vitamins. Renowned for their high nutritional value, health benefits, and accessibility, they have been a dietary staple for millions of years. Globally, rice, wheat, and maize contribute approximately 50% of the total calorie intake (Oliveira et al., 2017). Cereals are highly susceptible to fungal contamination at various stages, including cultivation, drying, processing, transportation, and storage. Preventing mycotoxin production in contaminated products is particularly challenging in tropical and subtropical climates, where high humidity and temperatures promote fungal growth and proliferation (V. Kumar et al., 2008).

Mycotoxins, toxic secondary metabolites produced by fungi, are heat-stable and can persist in various products. Contaminated cereals can lead to mycotoxin accumulation in animal and human organs, posing risks such as carcinogenic, mutagenic, genotoxic, teratogenic, neurotoxic, and estrogenic effects (Kamkar et al., 2014, Akrami Mohajeri et al., 2013). According to the Food and Agricultural Organization (FAO) reports, 25% of the world's agricultural products contain mycotoxins. In addition, about 5-10% of global food loss occurs due to fungal degradation. Multiple national and international public health organizations, including the FAO, the European Union (EU), and the World Health Organization (WHO), have set maximum levels for mycotoxins in foods like cereal and its derivatives (Oliveira et al., 2017).

In addition to mycotoxins, WHO has identified aflatoxins (AFs) as a global food safety hazard, with rural areas of developing nations being particularly at risk (Mehlhorn, 2016). As a consequence of their hazardous, carcinogenic, mutagenic, teratogenic and immunotoxic qualities, aflatoxins (AFs) were positioned in group 1 carcinogens by the International Agency for Research on Cancer (IARC) (Ostry et al., 2017). Aflatoxin molecules come in more than 20 different varieties, although aflatoxins B1 (AFB1), B2 (AFB2), G1 (AFG1), G2 (AFG2),

M1 (AFM1), and M2 (AFM2) are the most well-known (Ismail et al., 2018).

Aflatoxin exposure in humans occurs by eating foods contaminated with the toxin or by eating food made from an animal that has already been exposed to it (Leong et al., 2012). Both humans and animals who consume aflatoxins regularly may face serious health risks and life-threatening effects because of acute aflatoxin exposure. A harmful condition termed as "Aflatoxicosis" may result from the ingestion of aflatoxins through contaminated foods which may primarily affect the liver and lead to conditions such as hepatitis, cirrhosis, and an increased risk of liver cancer (Williams, 2004). Chronic exposure to aflatoxins weakens the immune system, making individuals more susceptible to infections, and may also cause gastrointestinal issues. In severe cases, acute aflatoxicosis can result in rapid liver failure, jaundice, lethargy, and, potentially, death. *Aspergillus flavus* and *Aspergillus parasiticus* are the common fungi that produce aflatoxins and may cause serious threat to human health including human hepatic malignancies, acute lethal disorders, and hepatic lesions (Murugavel et al., 2007).

In a study conducted among maize samples in Dinajpur district showed 56% contamination with *Aspergillus* spp. in fresh condition and 23.2% in the stored samples. (M Rafik et al., 2020). In another study conducted in Bangladesh in 2012, the Laboratory of Food Toxicology Research examined aflatoxin contamination in rice, wheat, and maize across six divisions for total aflatoxin B1, B2, G1 and G2. The study reported a high level (37%) of contamination and particularly 280µg/kg was found in maize(Nurul et al., 2013). Prior research in Bangladesh has predominantly focused on cereal grains such as rice, wheat, and maize. Notably, no previous study in Bangladesh has included processed cereals like corn flakes and oats. Our study is the first one in Bangladesh to incorporate processed cereal products like corn flakes, oats, wheat flour, and rice flour alongside the traditional cereal grains. Additionally, our study introduces a novel extraction method for aflatoxins, utilizing the QuEChERS technique, which distinguishes our study from others in the field.

Materials and Methods

Sample collection and processing

A total of 130 samples were collected from local markets around the areas of University of Dhaka. The samples included 5 brands of wheat flour (n = 5), 5 brands of corn flakes (n = 5), 2 brands of oats (n = 2), 3 brands of rice flour (n = 3), 6 types of locally available rice (n = 6), and 5 samples of corn (n = 5). Five packets of each brand were purchased from the local markets. Subsequently, all the packets were opened and combined. A pooled sample was then created from this amalgamation for each brand. After that, the pooled sample of 26 was examined for fungal identification and aflatoxin detection. The samples were collected from May 2023 to June 2023 and were kept in sterile zipper bags and stored in the refrigerator at 4◦C. The samples were used for preparation of inocula within 48 hours.

Morphological characterization of fungi

The fungi associated with the collected samples were isolated using the "Tissue Plating Method" (CAB 1968) on Potato Dextrose Agar (PDA) medium. Rice, corn, oats and corn flakes samples were directly placed on sterilized Petri plate containing PDA medium under aseptic condition. Each Petri plate contained 15 ml of PDA, one drop of lactic acid (0.03 ml), and five inocula. PDA was employed as a growing medium, and lactic acid was added to suppress growth of bacteria. Wheat flour and rice flour of each brand was combined with one milliliter of distilled water to create a semi-solid inocula. After preparing the semi-solid inocula, five of them were placed in a Petri plate containing PDA. A total of 6 Petri plates containing 30 inocula for each sample were used for one-time isolation. Inoculated Petri plates were incubated in the incubator at 25◦C temperature for seven days. After 7 days of incubation, colony characteristics such as color (observe, reverse), diameter, shape and sporulation were observed. Using a sterile scalpel, fungal structures such as mycelia, spore-bearing structures, and spores were scraped off from the surface and mounted in lacto phenol on a sanitized slide for microscopic examination. In the case of hyaline structures, a small amount of Cotton Blue (aniline blue) was added to the mounted fluid. A clean cover slip was placed over the material. Excess fluid was removed by soaking it with blotting paper and was placed under the microscope. The mycelia and spore observation were done at 40X magnifications.

The microphotographs of the fungi along with the measurement of spore size were taken by a high-resolution microscope facilitate with a camera (Nikon optiphot-2 trinocular microscope, Japan). The morphological identities of fungal isolates were determined using the standard literature. (Thom and Rapper,1945; Rapper and Thom, 1949; Ellis, 1971,1976; Booth, 1971).

The isolation frequency (Fr) and the relative density (RD) of genera were calculated according to the following formulas of Spurr and Wetly (1972).

$$frequency\ (\%) = \frac{\text{Number of samples of occurrence of a genus}}{\text{Total number of samples}} \times 100$$

$$relative\ density\ (\%) = \frac{\text{Number of isolates of a genus/species}}{\text{Total number of genus isolates}} \times 100$$

Molecular identification of Aspergillus flavus

DNA Extraction

Fungal cultures were grown on PDA medium at 25°C for 10 days. The fungal mycelium was harvested by scraping the surface of the 10-day-old cultures using a sterile spatula and collecting one gram into a 1.5 mL sterile Eppendorf tube. The mycelium was homogenized in 400 µL of sterile extraction buffer (200 mM Tris-HCl, 250 mM NaCl, 25 mM EDTA, 0.5% SDS) using a homogenizer. Subsequently, 6 µL of RNase (20 mg/mL) was added to each tube. The mixture was vortexed to achieve homogeneity and incubated in a 65°C water bath for 10 minutes. After incubation, the samples were cooled to room temperature, and 130 µL of 3M sodium acetate (pH 5.2) was added. The tubes were vortexed for 30 seconds at maximum speed and incubated at -20°C for 10 minutes. The samples were then centrifuged at 13,000 rpm for 15 minutes, and the supernatant was transferred to new tubes. An equal volume of chloroform:isoamyl alcohol (24:1) was added, mixed gently by inversion, and centrifuged at 12,000 rpm for 5 minutes. The supernatant was transferred to fresh tubes, and the chloroform:isoamyl alcohol extraction was repeated. The final supernatant was discarded, and the DNA pellet was washed twice with 700 µL of 70% ethanol.

The DNA pellet was air-dried in an oven at 40°C for at least 10 minutes before being resuspended in 100 µL of 1× TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). The DNA was allowed to dissolve overnight at 4°C and subsequently stored at -20°C for further analysis.

PCR Amplification

Molecular identification of the isolate was performed by amplifying the internal transcribed spacer (ITS) region. PCR amplification was conducted using the ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') as forward and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') as reverse primers. The PCR was carried out in a 0.2 ml PCR tube with 25 µl reaction volume containing 2.0 µl template DNA, 12.5 µl master mix, 1.0 µl forward primer, 1.0 µl reverse primer, and 8.5 sterile de-ionized distilled PCR water. The reaction mixture was vortexed and centrifuged in a microcentrifuge. The PCR was initiated by an initial denaturation step at 94◦C for 5 minutes following 30 cycles of 94◦C, 54◦C, and 72◦C each for 30 sec, with a final extension step of 5 min at 72◦C and ended with 4◦C. PCR amplified product was stored in a -20◦C freezer for analysis by resolving in 1% agarose gel. The gel was prepared using 1.0 g agarose powder containing ethidium bromide. Agarose gel electrophoresis was conducted in 1xTAE buffer at 90 Volts and 300 mA for 40 minutes. A gel documentation system (model: DI-HD, UK) was used to take pictures of the amplified PCR product.

Preparation of PCR product for sequencing

Before sending for sequencing, PCR product was purified by alcohol precipitation. After purification, the amplified DNA sample was diluted to a final concentration of 10-30 ng/µl before sending for sequencing. The quality and quantity of DNA samples were recorded using a Nanodrop. Then the DNA samples were sequenced through an automated sequencer in the Centre for Advanced Research in Sciences (CARS), University of Dhaka, Dhaka-1000.

Sequencing

Sequence alignment and editing were done with the BioEdit Sequence Alignment program and compared against the sequences already available in the databases using the program BLASTn (http://www.ncbi.nlm.nih.gov/BLAST).

Aflatoxin extraction and quantification

Rice, corn, oats, wheat flour, rice flour, and corn flakes samples were analyzed by using HPLC (High Performance Liquid Chromatography) with fluorescence detector and QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) extraction method (Sirhan et al., 2011), in Food Toxicology and Research Laboratory, IFST, Bangladesh Council of Scientific and Industrial Research (BCSIR).

Chemicals and materials

Aflatoxin standards were purchased from Sigma (St. Louis, MO, USA) with purity of 98%; standard stock solutions were prepared in acetonitrile according to the Association of Official Analytical Chemists (AOAC) method. HPLC-grade acetonitrile and methanol used for the mobile phase were purchased from Merck (Darmstadt, Germany), whereas analytical-grade acetonitrile and methanol used for extraction were purchased from Fischer Scientific (Leicestershire, UK). Water was purified by reverse osmosis followed by an electrodeionization (EDI) system (Maxima Ultra Pure Water, England). A 0.45 µm disposable membrane filter was purchased from Cronus Filter (UK). Anhydrous magnesium sulfate (MgSO4) and sodium chloride (NaCl) were purchased from Agilent Technologies (USA).

HPLC Analysis

The HPLC analyses were carried out with a JASCO FP-2020 Plus consisting of a degasser, tertiary pump, auto sampler, column oven and a fluorescence detector. The chromatographic separation was performed with a C18 150 mm × 4.6 mm Spherisorb 5 ODS-1 (particle size 5 µm) chromatographic column. The sample extracts were analyzed isocratically using 60:20:20 water/methanol/acetonitrile mixture as the mobile phase. The column was kept in a column oven at 30◦C at a flow rate of 1.0 mL/min to achieve the optimum resolution of the aflatoxins. The injection volume was maintained at 20 µL for both the sample and standard solutions.

Sample Preparation

In this study, the preparation of samples for aflatoxin detection closely followed the initial QuEChERS method with some modification (Sirhan et al., 2011), omitting any subsequent cleanup processes.

Step 1: A thoroughly homogenized sample (2.0 g) was weighed in a polypropylene centrifuge tube (15 mL).

Step 2: Methanol/acetonitrile/water mixture 51:9:40 (%, v/v) (10.0 mL) was added, and the centrifuge tube was manually shaken vigorously for 1min to ensure that the solvent mixed thoroughly with the entire sample for the complete extraction of the analyte.

Step 3: Anhydrous MgSO4 (2 g) and NaCl (1 g) were added to the mixture and the shaking procedure was repeated for 1 min in order to facilitate the extraction and partitioning of the four aflatoxins compounds into the organic layer.

Step 4: The extract was centrifuged for 5 min at 4000 rpm and subsequently 2.0 mL of the upper organic layer was filtered through a pipette.

Step 5: 0.075g PSA and 0.125 g C18 was added and shaken well.

Step 6: Centrifuged for 5 mins at 4000 rpm and subsequently 1.0 mL of the upper organic layer was filtered through a 0.45 µm syringe filter.

Results

Morphological identification

A total of 436 colonies and 9 fungal genera were formed from 156 isolation plates of wheat flour, rice, corn flakes, corn, oats, and rice flour samples. Aspergillus accounted for 38.99% of the relative density and was the most prevalent genus, occurring in 24 samples (92.31%) with 170 isolates. Sixty-nine isolates of Penicillium, representing a 15.82% relative density, were found in 19 samples (73.08%). There was a 16.05% relative density for Rhizopus, with 81 isolates found in 14 samples (53.85%). An 18.57% relative density of Fusarium was found in 4 samples (15.38%), totaling 70 isolates. Relative densities and frequencies were lower in other genera, such as Alternaria, Bipolaris, Curvularia, Trichoderma, and Cladosporium (Table 1).

Table 1. Frequency (Fr) and relative density (RD) of fungi in the cereal and cereal products samples.

Fungal genera isolated	Number of samples infected out of 26	Number of isolates	Frequency of isolation (%)	Relative density (%)
Aspergillus	24	170	92.31	38.99
Penicillium	19	69	73.08	15.82
Rhizopus	14	81	53.85	16.05
Fusarium	4	70	15.38	18.57
Cladosporium	3	37	11.53	8.48
Alternaria	3	6	11.53	1.37
Bipolaris	1	1	3.84	0.22
Curvularia	1	1	3.84	0.22
Trichoderma	1	1	3.84	0.22

19 fungal species were isolated from different samples tested. *Aspergillus flavus* (Raper), *Aspergillis niger* (Tiegh), *Aspergillus* sp.1, *Aspergillus* sp.2, *Aspergillus* sp.3, *Rhizopus stolonifer* (Ehrenb: Fr.), *Fusarium oxysporum* (Schltdl.), *Alternaria alternate* (Keissl), *Alternaria* sp.1, *Alternaria* sp.2, *Trichoderma viride* (Pers.), *Curvularia lunata* (Wakker), *Penicillium chrysogenum* (Thom), *Penicillium* sp.1, *Penicillium* sp.2, *Penicillium* sp.3, *Penicillium* sp.4, *Bipolaris* sp., *Cladosporium* sp. were identified morphologically through observing the characteristics (Figure 1).

Key morphological features of the isolated fungi

Aspergillus flavus var**.** Raper, K.B & Fennell, The Genus *Aspergillus*.:366 (1965) **Fig. 1(a)**

Colonies had olive-green conidia, leveled borders, elevated centers, rough hyaline conidiophores (up to 100 µm), globose vesicles (25-45 µm), and yellowish-green conidia (3-4 µm).

Specimen examined: Isolated from wheat flour, rice flour, corn, rice, and corn flakes samples from markets near Dhaka University, Bangladesh. 6 August, 2023. T. Akter 2.

Aspergillus niger Tiegh, Annales des Sciences Naturelles Botanique 8:240 (1867) **Fig. 1(b)**

Colonies were black and powdery with pale yellowish-white reverse; conidiophores arose from thick-walled brownish foot cells, with globose vesicles, double-series sterigmata, and rough, black, globose conidia (4-5 µm).

Specimen examined: Isolated from wheat flour, oats, corn, rice, corn flakes, and rice flour samples from markets near Dhaka University, Bangladesh. 6 August 2023. T. Akter 1.

***Aspergillus* sp.1 Fig. 1(c)**

A slow-growing fungus on PDA with grayish-white to yellowish-white colonies and globose conidia measuring 2–3 µm in diameter.

Specimen examined: Isolated from wheat flour sample from markets near Dhaka University, Bangladesh. 9 August, 2023. T. Akter 9.

***Aspergillus* sp.2 Fig. 1(d)**

Colonies white on PDA media. Conidial head globose. Sterigmata in double series. Conidia smooth walled, globose, 3-4 µm diameter.

Specimen examined: Isolated from wheat flour sample from markets near Dhaka University, Bangladesh. 8 August, 2023. T. Akter 8.

***Aspergillus* sp. 3 Fig. 1(e)**

Colonies cream colour at the top and yellowish white at reverse on PDA media. Conidia globose to subglobose, 3-4 x 2-3 µm diameter.

Specimen examined: Isolated from wheat flour sample from markets near Dhaka University, Bangladesh. 8 August, 2023. T. Akter 11.

Rhizopus stolonifer (Ehrenb: Fr.) Lind, Danish Fungi: 72, 1913 **Fig. 1(f)**

Colonies on PDA grow quickly, covering the plate in four days with black sporangia at the edges or across the dish. Brown, unbranched sporangiospores and rhizoid structures are visible at stolon and sporangiophore intersections

Specimen examined: Isolated from wheat flour samples, oats, corn, rice, corn flakes, and rice flour samples from markets near Dhaka University, Bangladesh. 13 August, 2023. T. Akter 4.

Fusarium oxysporum Schltdl. Flora berol. 2: 139, 1824; Fr., emend. Snyder & Hansen, Am. J. Bot. 27: 6067, 1940. **Fig. 1(g)**

Mycelium is fragile, typically purple, white, or peach, and felted in older cultures. Conidia are varied, with microconidia being ovoid to cylindrical and macroconidia having a thin-walled, pointed, and hooked shape.

Specimen examined: Isolated from corn samples from markets near Dhaka University, Bangladesh. 18 October, 2023. T. Akter 23.

Alternaria alternate (Fr.1832: Fr.) Keissl. 1912 **Fig. 1(h)**

Gray to olivaceous, powdery or felty colonies with unbranched, septate conidiophores (50 µm long) and pale brown, smooth to verrucose conidia, often with short conical or cylindrical beaks.

Specimen examined: Isolated from oats sample from markets near Dhaka University, Bangladesh. 18 October, 2023. T. Akter 21.

***Alternaria* sp.1 Fig. 1(i)**

Colonies rapidly expanding, black to grayish, and suede-like to floccose in texture. Conidia are obclavate to obpyriform, 4-10µm, pale brown, smooth or verrucose, often with short conical or cylindrical beaks.

Specimen examined: Isolated from rice and corn flakes samples from markets near Dhaka University, Bangladesh. 19 October, 2023. T. Akter 15.

***Alternaria* sp.2 Fig. 1(j)**

Dark brown to black colonies with septate, cylindrical conidiophores (up to 200 µm long, 4-7 µm wide) and smooth, brown to dark brown conidia, obclavate to obpyriform, with 3-7 transverse and occasional longitudinal septa.

Specimen examined: Isolated from oats sample from markets near Dhaka University, Bangladesh. 10 October, 2023. T. Akter 20.

Trichoderma viride Pers.: Fr. **Fig. 1(k)**

Colonies grow rapidly, starting hyaline and turning whitish-green with blue-green conidial tufts spreading from the margin to the center. Conidiophores are pyramidally branched at right angles, with (sub)spherical, hyaline, roughened conidia (3.6-4.5 µm in diameter).

Specimen examined: Isolated from wheat flour sample from markets near Dhaka University, Bangladesh. 10 September, 2023. T. Akter 7.

Curvularia lunata (Wakker) Boedijn - Bull. Jard. Bot. Buitenz., Ser. 3, 13: 127, 1933. **Fig. 1(l)**

Colonies are gray to blackish-gray with a black reverse, featuring erect, unbranched, septate conidiophores (37-64 µm long, 9.2-14.4 µm thick) with dark brown scars. Conidia are smooth, olivaceous brown, broadly clavate to obovoidal, 3-septate, and 24-31 × 8-11 µm, with paler end cells.

Specimen examined: Isolated from corn flakes sample from markets near Dhaka University, Bangladesh. 2 November, 2023. T. Akter 16.

Penicillium chrysogenum Thom, Bull. Bur. Anim. Ind. US Dept. Agric. (1910). **Fig. 1(m)**

Colonies reach 4-5 cm in 10 days, velvety to floccose, yellow-green with a yellow reverse. Monomorphic, verticillate conidiophores produce smooth-walled, hyaline to slightly green, ellipsoidal conidia (3.0-4.0 × 2.8-3.8 µm).

Specimen examined: Isolated from wheat flour, oats, corn, rice, corn flakes, and rice flour samples from markets near Dhaka University, Bangladesh. 8 November, 2023. T. Akter 3.

***Penicillium* sp.1 Fig. 1(n)**

Colonies are white, floccose, with high-domed mycelium and light white sporulation, pale on PDA media. Conidiophores are long (45-50 µm), with primarily terverticillate heads, metulae (8-14 × 2-5 µm), ampulliform phialides, and subglobose, slick conidia (3.50 µm).

Specimen examined: Isolated from wheat flour sample from markets near Dhaka University, Bangladesh. 30 October 2023. T. Akter 11.

***Penicillium* sp.2 Fig. 1(o)**

Colony growth of 15-20 mm in 7 days, with velutinous to slightly floccose, deep orange mycelium, substantial sporulation bordered by white, and a deep orange reverse; telerospores are heavy-walled, elliptical, and 18-20 µm in diameter.

Specimen examined: Isolated from rice sample from markets near Dhaka University, Bangladesh. 12 October, 2023. T. Akter 14.

***Penicillium* sp.3 Fig. 1(p)**

Colonies are dull yellow in the center with white peripheries, faintly fimbriated margins, and scarce sporulation; reverse brownish yellow. Conidia are smooth, globose to subglobose (2-3 µm), with short, mono- and biverticillate conidiophores, and vesicles 6-10 µm long.

Specimen examined: Isolated from corn flakes, and rice samples from markets near Dhaka University, Bangladesh. 15 November, 2023. T. Akter 13.

***Penicillium* sp.4 Fig. 1(q)**

Colony can be floccose, funiculose, or furrowed, with conidiophores branching near the apex and ending in phialides. Conidia are hyaline or brightly colored, single-celled, 3-4 µm, globose or ovoid, and arranged in basipetal chains.

Specimen examined: Isolated from rice flour sample from markets near Dhaka University, Bangladesh. 15 November, 2023. T. Akter 19.

***Bipolaris* sp. Fig. 1(r)**

Colonies are dark grey with white blotches, cottony, 2-6 mm, flat, densely floccose, and a deep black reverse. Conidia are large, curved, fusoid, with 6-9 tiny septa, smooth, dark, thick walls, 20-30 x 6-10 µm.

Specimen examined: Isolated from oats sample from markets near Dhaka University, Bangladesh. 12 September, 2023. T. Akter 22.

***Cladosporium* sp. Fig. 1(s)**

Colonies are olivaceous to blackish brown with abundant sporulation, flat and wrinkled centers, and a black olivaceous reverse. Conidiophores are upright, with smooth to verrucose conidia (5-10 x 2-3 µm) and a dark hilum.

Specimen examined: Isolated from rice sample from markets near Dhaka University, Bangladesh. 26 September, 2023. T. Akter 14.

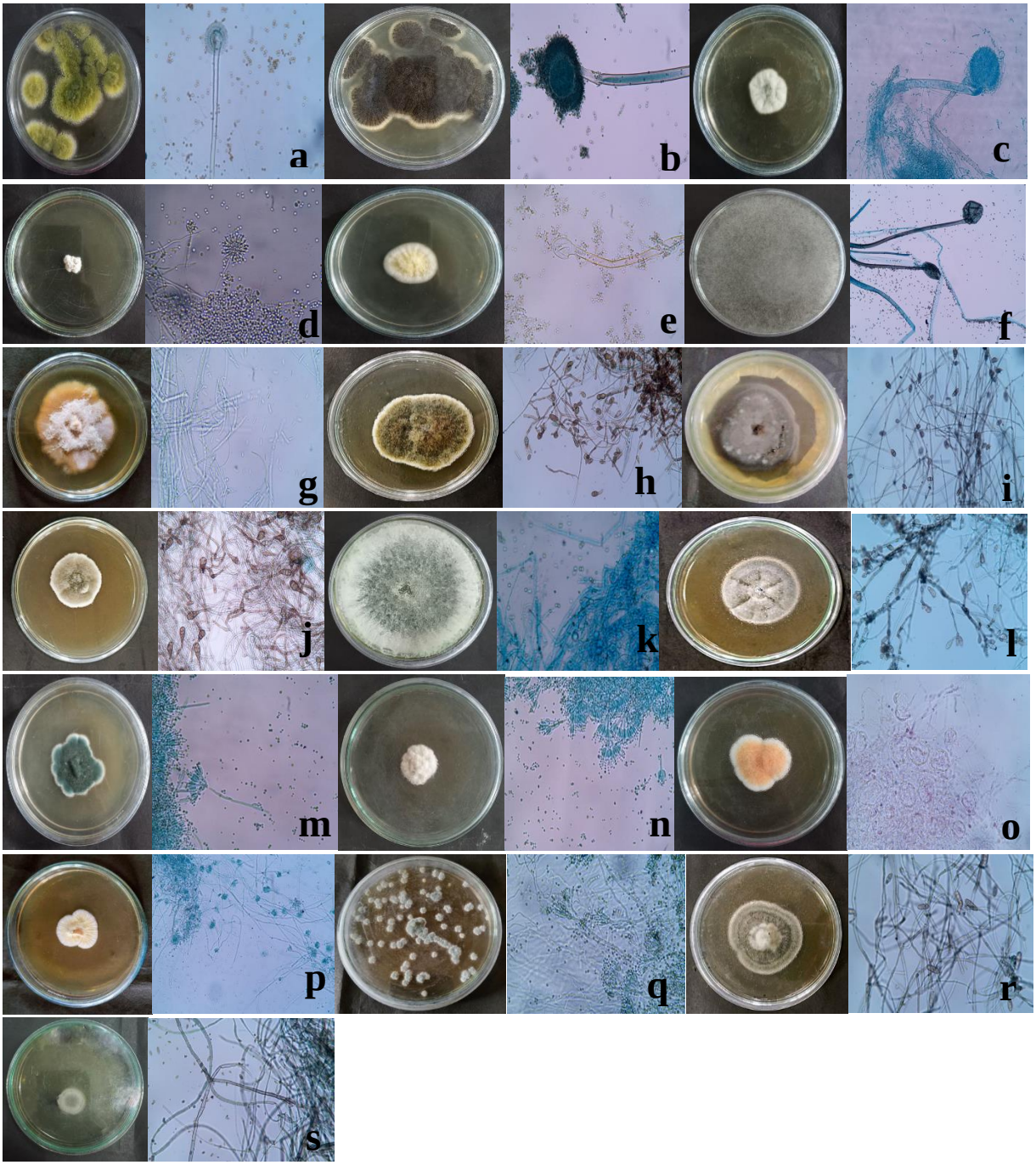

Figure 1. Colony of the isolates on PDA medium and conidia under microscope **a** *Aspergillus flavus*, **b** *Aspergillus niger*, **c** *Aspergillus* sp.1, **d** *Aspergillus* sp.2, **e** *Aspergillus* sp.3, **f** *Rhizopus stolonifer*, **g** *Fusarium oxysporum*, **h** *Alternaria alternate*, **i** *Alternaria* sp.1, **j** *Alternaria* sp.2, **k** *Trichoderma viride*, **l** *Curvularia lunata*, **m** *Penicillium chrysogenum*, **n** *Penicillium* sp.1, **o** *Penicillium* sp.2, **p** *Penicillium* sp. 3, **q** *Penicillium* sp.4, **r** *Bipolaris* sp., **s** *Cladosporium* sp.

Molecular identification of *Aspergillus flavus*

PCR amplification of the ITS region of the isolate using ITS1 and ITS4 primers produced a distinct band of approximately 550 bp on a 1% agarose gel (Figure 2), indicating the successful amplification of the target DNA fragment. Sequence analysis of the amplified ITS region using the Basic Local Alignment Search Tool for nucleotides (BLASTn) revealed 100% identity with *Aspergillus flavus* sequences available in the National Center for Biotechnology Information (NCBI) database (Table 2). These results confirm the molecular identification of the isolate as *Aspergillus flavus.*

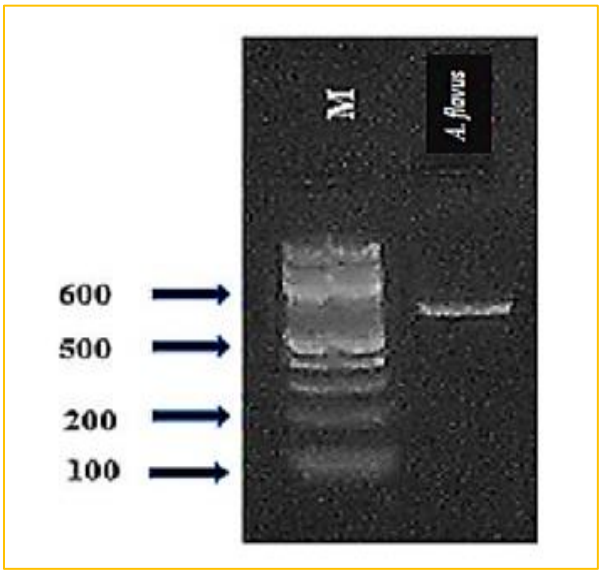

Figure 2. Gel electrophoresis of the PCR product of the isolated *Aspergillus flavus* performed by ITS1and ITS4 primers and showing ~550 bp amplification (M represents 1kb DNA ladder).

Table 2. BLAST analysis of the amplified sequences from the isolated DNA of *Aspergillus flavus.*

Name of Fungi	Max score	Total Score	Query coverage	E value	Identity (%)	NCBI Gene Bank Accession No.
Aspergillus flavus	1197	2100	100%	0.0	100%	MK645222

Determination of aflatoxins in cereals and cereal product samples

In total, 77.03% of the samples tested positive for aflatoxins, with an average concentration of 2.17 µg/kg. Aflatoxin B1 was detected in 34.61% of the samples, with concentrations ranging up to 8.9 µg/kg. Aflatoxin B2 was found in 11.53% of the samples, with concentrations up to 1.5 µg/kg. Aflatoxin G1 was present in 11.53% of the samples, with concentrations up to 2.57 µg/kg, while Aflatoxin G2 appeared in 30.77% of the samples, with concentrations up to 2.32 µg/kg (Table 3).

Table 3. Aflatoxin (B1, B2, G1, G2) levels found in cereals and cereal product samples.

Aflatoxins	Positive sample (%)	Min – Max Concentration (µg/kg)	Mean ± SD (µg/kg)	LOD (µg/kg)
B1	34.61	ND*-8.9	2.397 ± 2.562	0.5
B2	11.53	ND-1.5	0.78 ± 1.018	0.15
G1	11.53	ND-2.57	1.8 ± 1.024	0.5
G2	30.77	ND-2.32	1.157 ± 0.861	0.15
Total Aflatoxins (B1, B2, G1, G2)	73.03	ND-8.9	2.17± 1.87	0.15-0.5

*ND= Not Detected

Among the tested samples, 19 were positive for aflatoxin residues, indicating aflatoxin contamination. Two samples exceeded the Maximum Residue Limit (MRL) of aflatoxin B1(2 µg/kg) and total aflatoxins (4 µg/kg) as per Commission Regulation (EC). Wheat flour sample 3 exhibited an aflatoxin concentration with a total aflatoxin level of 3.05 µg/kg, exceeding the Maximum residual limit (MRL) for aflatoxin B1. Corn flakes sample 2 also exceeded the MRL, with aflatoxin B1 and total aflatoxin concentrations of 8.9 µg/kg. The remaining positive samples had aflatoxin concentrations below the MRLs, ranging from 0.2 to 3.76 µg/kg. Aflatoxin was not identified in seven samples, suggesting trace amounts below the detection limits (Figure 3).

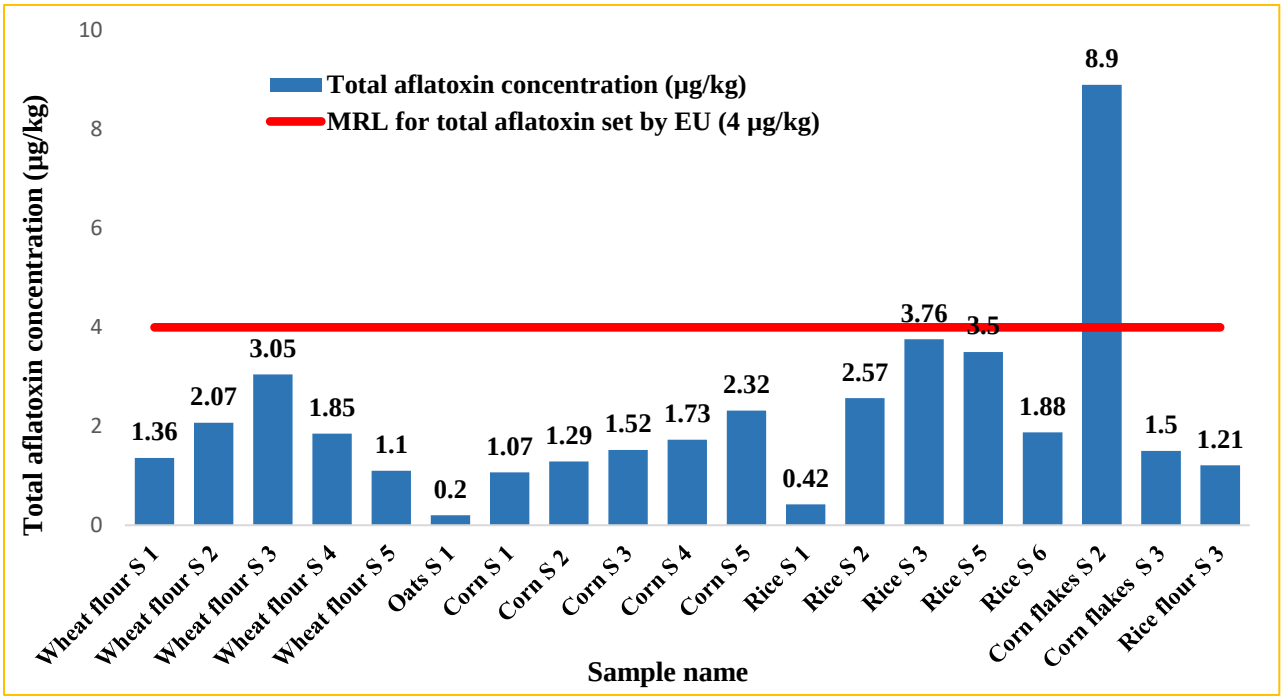

Figure 3. Comparison of total aflatoxin concentrations (µg/kg) in various cereal-based products (blue bars) with the Maximum Residue Limit (MRL) for total aflatoxin set by the European Union (4 µg/kg, red line). Samples include wheat flour, oats, corn, rice, corn flakes, and rice flour from various sources. Most samples have aflatoxin levels below the EU MRL, except for one corn flakes sample (Corn flakes S2) which significantly exceeds the limit (8.9 µg/kg).

Discussion

Cereals and cereal products are highly consumed throughout the world due to their nutritional value and convenience. In both developed and developing nations, the consumption of processed cereals such as corn flakes, oats, wheat flour and rice flour is increasing steadily. The present study focused on assessing the level of fungal contamination and the detection of aflatoxin residues in various cereal products, including wheat flour, corn flakes, oats, corn, rice flour, and rice samples. The study identified 19 fungal species, including *Aspergillus*, *Penicillium*, *Fusarium*, *Trichoderma*, *Rhizopus*, *Alternaria*, *Curvularia*, *Bipolaris*, and *Cladosporium*. Among these, *Aspergillus*, particularly *Aspergillus flavus*, and *Penicillium chrysogenum* were found to be the most prevalent in almost all tested products. The presence of potentially toxigenic fungi in the samples is a matter of concern since certain species can produce mycotoxins such as aflatoxins, fumonisins, trichothecenes, zearalenone, patulin, and citrinin (Piotrowska, 2013). The study revealed that 92.31% (24/26) of the samples were contaminated with *Aspergillus* species, highlighting the potential risk of aflatoxin contamination in these cereal products. Inadequate storage conditions, improper harvesting techniques, suboptimal crop management, inadequately controlled processing methods, climate conditions favoring fungal growth, transportation and post-harvest storage challenges, possible seed contamination, insect infestation, a lack of awareness and implementation of mycotoxin management programs, and inadequate monitoring and regulatory measures in the production and distribution chain may be some of the factors contributing to the high incidence of Aspergillus species contamination. These findings are consistent with previous research indicating that these fungal species are common contaminants in cereals and cereal products which were done outside of Bangladesh (Hussaini et al., 2007; Tournas & Niazi, 2018). *Fusarium oxysporum* was found to be more prevalent in corn samples, highlighting the global issue of *Fusarium* contamination in corn (Mislivec et al., 1979, Tournas & Niazi, 2018). Findings of a study conducted in Bangladesh revealed that 56% of maize samples exhibited contamination with Aspergillus spp. in fresh samples and 23.2% in stored ones. Notably, 20 samples exceeded the 20 ppb aflatoxin threshold. However, the study focused exclusively on maize samples, whereas our research spans both cereal grains and cereal products. Remarkably, fungal identification and aflatoxin detection have been insufficiently explored within Bangladesh, with the majority of studies conducted beyond its borders and limited to cereal grains (rice, wheat, maize) (Nurul et al., 2013). Significantly, there has been a notable absence of

prior examinations on corn flakes and oats within Bangladesh, despite the increasing consumption of these products. This oversight is especially significant considering the conducive weather conditions in the country that facilitate the growth of fungi. Therefore, our study on the presence of fungi and aflatoxins in cereals and cereal products assumes great importance within the context of Bangladesh. It aims to address the existing gap in research and provide insights into the evolving dietary patterns in the country.

Aflatoxins, in particular, are known to be highly carcinogenic and can lead to liver damage, immune suppression, and growth retardation. The detection of aflatoxin residues in the tested samples further emphasized the presence of mycotoxins in the food supply. Out of the 26 samples analyzed, 19 were found to contain aflatoxins B1, B2, G1, and G2. Two samples exceeded the maximum residue limit (MRL) set by the European Commission for aflatoxin B1 and total aflatoxins. Though these findings are consistent with previous studies that have reported aflatoxin contamination in various cereal-based products (Lee et al., 2017) in our study a new method (QuEChERS) for aflatoxin extraction was incorporated. Our study in Bangladesh differed from previous practices by employing the QuEChERS method for aflatoxin detection in cereals and cereal products, a methodology not previously utilized in this context (Nurul et al., 2013). The identification of *Aspergillus flavus* as the main fungi responsible for aflatoxin production underscores the importance of monitoring and controlling this specific species. The molecular identification of *Aspergillus flavus* confirmed its presence in the tested samples, further supporting the link between *Aspergillus* species and aflatoxin production. The high prevalence of aflatoxin residues in cereal products raises significant health concerns, especially considering that these products are widely consumed worldwide. Aflatoxin B1, in particular, has been associated with the development of hepatocellular carcinoma, a form of liver cancer, in regions where hepatoma is prevalent. It is crucial for regulatory agencies and food safety authorities to address the issue of mycotoxin contamination in cereals and cereal-based products. Strict adherence to maximum residue limits and implementation of effective monitoring and control measures are essential to minimize the risk of mycotoxin exposure through food consumption. In conclusion, the findings of this study highlight the need for continuous monitoring and control of fungal contamination and mycotoxin levels in cereals and cereal-based products. The presence of potentially toxigenic fungi and aflatoxin residues emphasizes the importance of ensuring food safety and protecting public health. Implementing appropriate measures to reduce fungal contamination and mycotoxin production is crucial to safeguarding the quality and safety of these widely consumed food items.

The study included a wide variety of food products (rice, corn, oats, corn flakes, wheat flour, and rice flour), indicating a comprehensive approach to understanding fungal contamination and aflatoxin presence in various staples and providing a more holistic view of potential aflatoxin contamination sources in the diet. The identification of aflatoxin in foods that are routinely consumed can directly affect public awareness and food safety regulations. The focus on isolating and identifying fungi in this study is essential for understanding the specific contaminants involved, contributing to a more accurate assessment of the potential health risks. The outcome of the study would help to increase public awareness, promote economic stability, protect public health, and develop evidence-based policy regarding food safety. However, this study had some drawbacks, such as limited sample size, absence of risk assessment, lack of control measures, and so on. It should be noted, however, that this study focused primarily on the detection of aflatoxins and did not examine other mycotoxins that may be present in these cereal products. More research should be done to determine the prevalence of other mycotoxins and their possible health hazards. Only the presence of *Aspergillus flavus* was identified in this study using morphological and molecular characteristics. Other fungal species found in this work can be validated by molecular characterization as well.

In conclusion, this study demonstrates a high level of fungal contamination and the presence of aflatoxin residues in various cereals and cereal-based products. The prevalence of toxigenic fungi, particularly *Aspergillus* species, raises concerns about the potential health risks associated with consuming contaminated cereals. The detection of aflatoxins, including the exceedance of the maximum residue limit in some samples, further highlights the need for effective monitoring and regulation to ensure consumer safety. The findings of this study emphasize the importance of implementing strict quality control measures and enforcing regulatory standards to prevent mycotoxin contamination in cereals and cereal products. Further research is needed to investigate the presence of other mycotoxins in these samples, as not all mycotoxins were detected in this study. Continued efforts to raise awareness about mycotoxin contamination and its health implications are crucial to protecting public health and promoting food safety in the global cereal market.

Conclusion

This study highlights significant fungal contamination and aflatoxin residues in cereals and cereal-based products, with a particular concern about Aspergillus species. The detection of aflatoxins, some exceeding the maximum residue limits, underscores the need for better monitoring and regulation to protect consumer health. The findings stress the importance of enforcing strict quality control measures and regulatory standards to prevent mycotoxin contamination. Further research is necessary to explore other mycotoxins, and efforts to raise awareness about their health risks are essential for promoting food safety globally.

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