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Research Article

REVELATION OF ANTIFUNGAL SUSCEPTIBILITY OF BOTANICALS AND GENETIC DIVERSITY OF AFLATOXIN-PRODUCING *ASPERGILLUS FLAVUS* IN CHILI (*CAPSICUM ANNUUM*)

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ABSTRACT

Aflatoxins associated with *Aspergillus flavus* pose a significant risk to food safety and public health. This study determined the minimum inhibitory concentration (MIC) of five medicinal plant extracts against *A. flavus*. Molecular identification, performed by amplifying the ITS rDNA region, confirmed the isolate as *A. flavus* (99.80% similarity to the GenBank sequence OP480002.1). The antifungal activities of botanical extracts (*Piper betle, Centella asiatica, Azadirachta indica, Mentha spicata,* and *Ocimum tenuiflorum*) at different concentrations (600 mg/ml, 300 mg/ml, and 100 mg/ml) were evaluated using disc diffusion and broth dilution methods, with Fluconazole as a reference. Except for *M. spicata*, all extracts exhibited inhibitory activity, with *A. indica* showing the highest inhibition (19.83 mm at 600 mg/ml), surpassing Fluconazole (15.67 mm). The MIC values were lowest for Fluconazole (4.68 mg/ml), followed by *A. indica* (9.375 mg/ml). RAPD-PCR analysis revealed significant genetic diversity among isolates, with 12 primers generating 50 polymorphic bands. Genetic similarity ranged from 73% to 99.2%, with the highest diversity observed between isolates AS3 and AS4. These findings highlight the potential of these medicinal plant extracts as natural alternatives to synthetic fungicides at specific concentrations for reducing aflatoxin contamination in agricultural products. Furthermore, the observed genetic diversity may help in assessing the aflatoxin production capacity of *A. flavus* in different chili cultivars.

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INTRODUCTION

Dried red chilies (*Capsicum* spp.) of the Solanaceae family are an essential spice and a significant cash crop in Bangladesh, where exports have increased by 67 percent. In Southeast Asia and Sub-Saharan Africa, more than 80% of chilies are produced, with a substantial portion affected by aflatoxin contamination (FAOSTAT 2017). Temperature and humidity play crucial roles in influencing toxin production both before and after harvest. Factors such as plant stress (due to insect and drought damage), warm temperatures (above 28℃), delayed harvesting, late irrigation, and the presence of rain or dew during warm periods are associated with crop infection and elevated aflatoxin levels (Hossain et

al. 2018; Singh and Cotty, 2019).

Aflatoxin-producing *Aspergillus flavus* is well known for its ability to infiltrate and colonize a wide range of stored agricultural commodities, including oilseeds, grains, wheat, chilies, and nuts (Jannat et al., 2022). South Asia currently has the highest prevalence of aflatoxin contamination globally, reaching an alarming rate of 82% (Jannat et al., 2022). Aflatoxin levels of 10-20 µg/kg have been detected in whole (27.9%), crushed (27.8%), and powdered (28%) chilies (Khan et al., 2014). Aflatoxin contamination in grains and other agricultural products poses a significant threat to food safety (Szabo et al., 2018). This underscores the need for continuous efforts to mitigate the impact of mycotoxin contamination on the food supply (Behiry et al., 2022).

Efforts to mitigate mycotoxin contamination involve both chemical and microbiological approaches (Adebiyi et al., 2019). However, aflatoxin contamination and the chemical exposure associated with its mitigation are twin challenges in the global market. Chemical methods, such as the use of ammonia, sodium hydroxide, and other substances, often fail to achieve complete decontamination and raise concerns regarding residual toxicity, environmental impact, and adverse effects on the nutritional and sensory qualities of food (Makhuvele et al., 2020). Consequently, there is growing interest in plant-based alternatives to synthetic fungicides for controlling fungal growth and aflatoxin production (Shahi et al., 2003). Botanical extracts, herbs, and essential oils are considered safer and cost-effective alternatives, as they provide bioactive phytochemicals that act as synergistic protectants and biofungicides (Kavitha et al., 2020; Prakash et al., 2020). The compounds in the plant extracts may serve as protectants against pathogens due to their potential antigenotoxic and antimicrobial properties, contributing to the development of a sustainable management practice for controlling aflatoxin contamination in chilies under field conditions (Makhuvele et al., 2020).

For better suppression of aflatoxin-producing fungi and to achieve long-term reduction in contamination, identifying fungal strengths and conducting genetic diversity analysis are essential. The species consists of two main morphotypes, L and S strains, with L morphotype fungi producing significantly less aflatoxin (mean = 43 µg/g) than S morphotype fungi (mean = 667 µg/g; $p < 0.01$) in liquid fermentation (Singh and Cotty. 2019). Assessing genetic diversity helps in identifying aflatoxigenic genotypes for potential biocontrol. One of the most frequently used genotyping methods for recognizing and characterizing fungal species is random amplified polymorphic DNA (RAPD) analysis (Chongo et al., 2004). Before using the RAPD method for epidemiological surveys, it is necessary to evaluate the extent of the differences in DNA patterns that can be generated.

The present study aimed to develop an integrated strategy for managing aflatoxin contamination in chili by identifying the fungal pathogen, determining the minimum inhibitory concentration of five medicinal plant extracts namely neem (*Azadirachta indica*), betel (*Piper betle*), sacred basil (*Ocimum tenuiflorum*), spearmint (*Mentha spicata*), and Asiatic pennywort (*Centella asiatica*) in comparison to the fungicide fluconazole, and evaluating the genetic diversity of *Aspergillus flavus* morphotypes associated with chilies under *in vitro* conditions.

MATERIALS AND METHODS

Collection of chili samples and isolation of fungi

A total of 50 samples from five different cultivars of infected chili (*Capsicum annuum* L.) were collected from various grocery stores in Khulna and Dhaka districts, Bangladesh. Twelve *A. flavus* isolates were obtained following the method described by Kennedy (2022). Molecular identification was performed to confirm the isolates.

DNA extraction

The *Aspergillus* species were cultured in potato dextrose broth and incubated for five days with continuous shaking at 150 rpm. After incubation, 20 g of mycelium was harvested for DNA extraction. The PureLink Fungal DNA Purification Kit (Invitrogen, Life Technologies, USA) was used for DNA extraction following the manufacturer's protocol. The purified DNA was quantified using a spectrophotometer (Multiskan GO Microplate, Thermo Fisher Scientific, Germany) at a wavelength of 260 nm.

Molecular identification based on internal transcribed spacers (ITS)

The internal transcribed spacer (ITS) regions of the twelve *A. flavus* isolates were amplified using the universal eukaryotic forward primer ITS5 (GGAAGTAAAAGTCGTAACAAGG) and reverse primer ITS4 (TCCTCCGCTTATTGATATGC) (Table 1).

Table 1. Composition of polymerase chain reaction chemicals for molecular identification.

Sr. No.	PCR reaction chemicals	Concentration	Quantity (µl)
1	Genomic DNA	50 ng μL^{-1}	1.0
2	Reaction buffer without $MgCl_2$	10X	2.5
3	dNTPs	10mM	0.5
4	Universal eukaryotic forward primer ITS5	5 pM μL^{-1}	0.5
5	Universal eukaryotic reverse primer ITS4	5 pM μL^{-1}	0.5
6	Tag DNA polymerase	5 U μL^{-1}	0.25
7	$MgCl_2$	25 mM	1.5
8	Molecular water		18.25
	The total volume of the reaction mixture		25.0

Collection of medicinal plant materials

Fresh, healthy leaves of *A. indica*, *P. betel*, *C. asiatica*, *O. tenuiflorum*, and *M. spicata* were collected from the local market in Khulna District.

Extraction of plant material and sample preparation

The selected plant materials were washed, shade-dried, and ground into a fine powder using a grinder. Two hundred grams of each powdered sample were soaked in 500 ml of methanol for 48 h, with hourly stirring using a sterile rod. After incubation, the extracts were filtered through Whatman No. 1 filter paper. The filtrates were then evaporated using a rotary evaporator and freeze-dried at 4°C to obtain a semi-solid residue (Njoki et al., 2017).

The obtained residues were precisely weighed in amounts of 100 mg, 300 mg, and 600 mg. Each portion was mixed with 1 ml of dimethyl sulfoxide (DMSO) using a vortex mixer and a sonicator to ensure a uniform solution. The final stock solution concentrations were 100 mg/ml, 300 mg/ml, and 600 mg/ml per extract. Moreover, 150 mg of fluconazole was dissolved in 1 ml of DMSO to obtain a standard concentration of 150 mg/ml.

Preparation of fungal spore suspension

Spores from four-day-old PDA cultures incubated at 28°C were suspended in sterile distilled water containing 0.9% NaCl and three drops of Tween 80. The suspension was standardized to 0.5 McFarland using a densitometer and spectrophotometer (absorbance: 0.08-0.12 at 625 nm), corresponding to a concentration of approximately 1×10^8 CFU/ml (Maida et al., 2008).

Measurement of inhibition zone using the disc diffusion method

Fungal spore suspensions (~1×10^8 CFU/ml) were inoculated onto potato dextrose agar (PDA) plates using sterile swabs (Maida et al., 2008). The PDA plates were divided into four sections: two for the test samples (positive control), one for the standard, and one for the blank (negative control) (Figure 1). Sample, standard, and blank discs were applied accordingly. Discs soaked in stock solutions were dried before being placed on the inoculated media, then incubated at 25°C for 72 h (Chukwudi and Ezeabara, 2017). Antifungal activity was assessed by measuring the inhibition zone using slide calipers on a non-reflective surface under reflected light, as described by Hudzicki (2009). The clear zone diameter was recorded in millimeters.

Determination of minimum inhibitory concentration (MIC)

Eight Eppendorf tubes (labeled 0-7) were prepared, with tubes 1-7 containing 0.5 ml of potato dextrose broth and tube 0 containing 1 ml of the extract solution. Tube 7 served as the sterile growth control. A two-fold serial dilution was performed by transferring 0.5 ml from tube 1 to tube 6. Each tube was then inoculated with 0.2 ml of a fungal spore suspension (adjusted to the 0.5 McFarland standard), yielding final extract concentrations of 300 mg/ml, 150 mg/ml, 75 mg/ml, 37.5 mg/ml, 18.75 mg/ml, 9.375 mg/ml, and 4.6875 mg/ml. For Fluconazole, the final concentrations were 100 mg/ml, 50 mg/ml, 25 mg/ml, 12.5 mg/ml, 6.25 mg/ml, 3.125 mg/ml, and 1.5625 mg/ml. The tubes were incubated at 29°C for 72 h, after which 50 µg of Resazurin was added to assess cell viability (Di Ciaccio et al., 2020). The MIC was determined as the lowest concentration of extract that exhibited no visible growth, indicated by a blue color, for *A. flavus*.

Genetic diversity analysis using RAPD primers

Genetic diversity was analyzed using 12 random primers. The RAPD analysis through PCR followed the same composition as the ITS analysis, except for the primers (Table 2).

Table 2. Primers and their corresponding sequences.

Sr. No.	Primer Code	Primer Sequence (5´- 3´)
1	OPA-18	AGGTGACCGT
2	OPF-04	TGCGCCCTTC
3	OPF-05	GGTGACGCAG
4	OPM-04	TGAGACCCGT
5	OPM-10	CCACAGCAGT
6	OPM-12	AGGGAACGAG
7	OPP-04	ACCCCGCCAA
8	OPW-02	GGCGGATAAG
9	OPW-05	TGCGGATAAG
10	UBC-25	GTGTCTCAGG
11	UBC-155	CTGGCGGCTG
12	UBC-173	CAGGCGGCGT

PCR amplification and gel documentation

PCR products were analyzed using an initial denaturation step at 94°C for 2 min, followed by 40 cycles of denaturation at 95°C for 30 sec, primer annealing at 32°C for 1 min, and primer extension at 72°C for 1 min. A final extension was performed at 72°C for 5 min.

For the quantification of amplified sequences, a 1 kbp DNA ladder (Direct Load, Sigma-Aldrich, USA) was used. The PCR products were separated via electrophoresis on a 1% agarose gel. Ethidium bromide (0.5 µg/mL) was used for staining. The agarose gel was prepared in 1.0× Tris-Acetate-EDTA (TAE) buffer and run at 60 volts for 1 h. The amplified DNA bands were visualized using a gel documentation system (BioDoc Analyze 2.2, Germany).

PCR product analysis

For genetic diversity analysis, 12 isolates were examined, with one primer used for each isolate. All data were replicated three times to minimize errors. Each sample profile was analyzed based on binary data, where the presence (1) and absence (0) of bands represented RAPD product patterns.

A phylogenetic tree was constructed using the Parsimony method (PAUP) to determine genetic relationships. A pairwise distance matrix was used to generate a dendrogram following the unweighted pair group method with arithmetic mean (UPGMA) described by Sneath and Sokal (1973). Additionally, the neighbor-joining (NJ) method (Saitou and Nei, 1987) was used for cluster analysis to further illustrate genetic relationships.

Statistical analysis

Each measurement was replicated three times for each treatment, and the data were reported as mean ± SE (standard error). A one-way analysis of variance (ANOVA) was performed to analyze the data, and differences among means were evaluated for significance at $p < 0.05$ using the least significant difference (LSD) test in Statistix 10 software. The functional relationship between botanical concentration and inhibition frequency was analyzed using Microsoft Excel.

RESULTS

Detection of *A. flavus* using the PCR method

The ITS region of the isolates was amplified and sequenced, yielding a 503-base pair fragment. A Basic Local Alignment Search Tool (BLAST) analysis of the ITS rDNA sequences in the National Center for Biotechnology Information (NCBI) GenBank identified the isolates as *A. flavus*, showing 99.80% similarity to the reference sequence OP480002 (Table 3).

Table 3. ITS rDNA sequence of *A. flavus*.

Species Name	ITS rDNA sequence	Similarity Percent	ITS rDNA Length (bp)
Gene Bank Id: OP480002.1 *Aspergillus flavus*	GATGGGCAGCAATGACGCTCGGACAGGCATGCCCCCCGGAATACCAG GGGGCGCAATGTGCGTTCAAAGACTCGATGATTCACGGAATTCTGCA ATTCACACTAGTTATCGCATTTCGCTGCGTTCTTCATCGATGCCGGAA CCAAGAGATCCATTGTTGAAAGTTTTAACTGATTGCGATACAATCAA CTCAGACTTCACTAGATCAGACAGAGTTCGTGGTGTCTCCGGCGGGCG CGGGCCCGGGGCTGAGAGCCCCCGGCGGCCATGAATGGCGGGCCCGCC KAAGCAACTAAGGTACAGTAAACACGGGTGGGAGGTTGGGCTCGCTA GGAACCCTACACTCGGTAATGATCCTTCCGCAGGTTCACCTACGGAAA CCTTGTTAC	99.80	503

Antifungal activity of botanical extracts

Determination of the zone of inhibition

The evaluation of crude medicinal plant extracts of *A. indica*, *C. asiatica*, *P. betle*, and *O. tenuiflorum* for their efficacy in inhibiting *A. flavus* revealed varying degrees of inhibitory potential at different concentrations (600 mg/ml, 300 mg/ml, and 100 mg/ml).

In this experiment, four out of five plant leaf extracts, with the exception of *M. spicata*, exhibited antifungal activity against aflatoxin-producing *A. flavus*. *A. indica* demonstrated substantial inhibitory effects, with a zone of inhibition of 19.83 ± 0.31 mm at 600 mg/ml, outperforming Fluconazole, which exhibited a zone of inhibition of 15.67 ± 0.00 mm at 150 mg/ml. At the same concentration (600 mg/ml), *P. betle* displayed a statistically similar inhibitory effect (14.50 ± 0.76 mm), comparable to both Fluconazole and *A. indica* at 300 mg/ml (14.67 ± 0.33 mm), highlighting its noteworthy antifungal activity (Figure 1).

C. asiatica at 600 mg/ml showed an inhibition zone of 11.33 ± 0.33 mm, which was closely related to the inhibitory effect of *P. betle* at 300 mg/ml. This activity was quite satisfactory in comparison to the inhibition zone of Fluconazole (150 mg/ml). *O. tenuiflorum* exhibited inconsistent results, showing an inhibition zone of 9.17 ± 0.31 mm at 600 mg/ml, followed by reduced inhibition at 300 mg/ml and 100 mg/ml. *M. spicata* did not exhibit significant antifungal activity. A significant ($p < 0.05$) difference was detected in the inhibition zones of *A. indica* at different concentrations (Table 4).

Upon reducing the concentration to 100 mg/ml, *A. indica* maintained significant antimicrobial activity, with an inhibition zone of 10.67 ± 0.42 mm. *P. betle* exhibited an inhibition zone of 8.67 mm, while *C. asiatica* demonstrated consistent efficacy (9.17 ± 0.31 mm). At 100 mg/ml, the inhibition zones produced by *P. betle* and *C. asiatica* were significantly different ($p < 0.05$) compared to Fluconazole. Interestingly, at this lower concentration, *O. tenuiflorum* and *M. spicata* did not display any inhibitory activity.

Functional relationship between the concentration of botanicals and their inhibitory activity

The functional relationship between different concentrations of plant extracts and their inhibition zones is shown in Figure 2. The equation for linear regression indicated a direct correlation. *A. indica* leaf extract exhibited concentration-dependent inhibition, with a substantial inhibition zone of 19.83 mm at 600 mg/ml and a strong positive correlation (R^2 = 0.9982) between concentration and inhibition zone size. *Pl betle* showed an inhibition zone of 14.50 mm at the same concentration, approaching the efficacy of Fluconazole (15.67 mm). *C. asiatica* also displayed a positive correlation between concentration and inhibition zone size, with an R^2 value of 0.8875. In contrast, *O. tenuiflorum* exhibited reduced antifungal activity at lower concentrations, with no inhibition zone observed at 100 mg/ml. These results highlight the potential of natural leaf extracts as alternative antifungal agents, particularly at higher concentrations, with *P, betle* and *A, indica* extracts demonstrating promising efficacy.

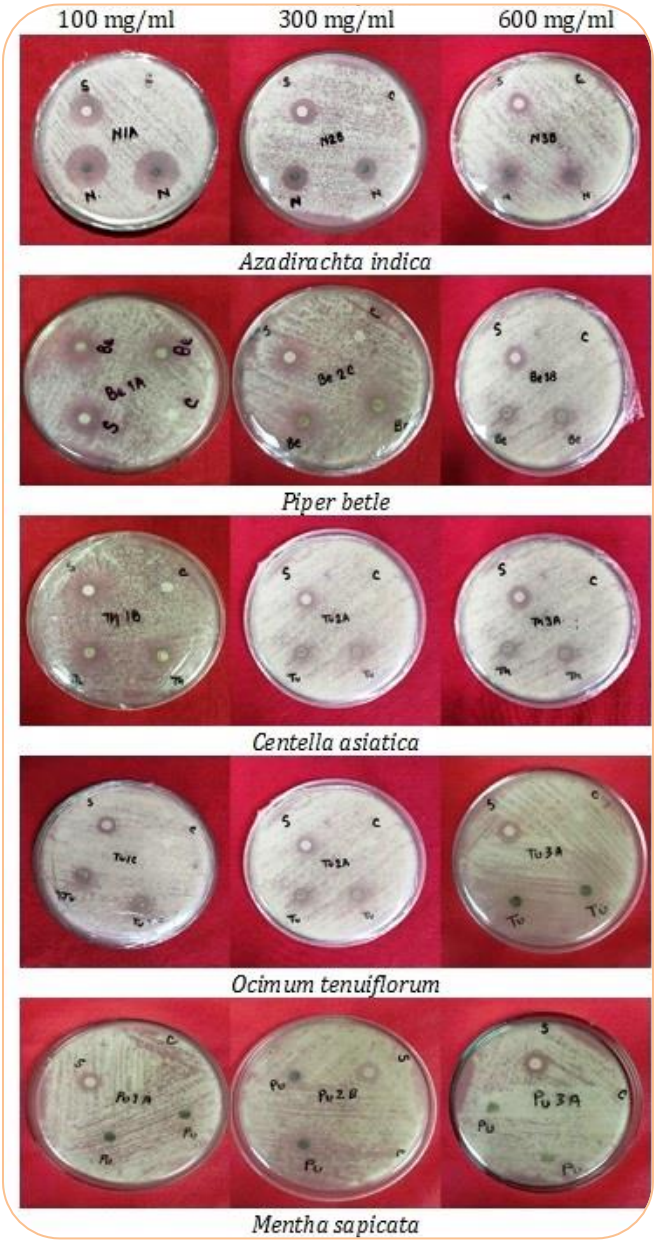

Figure 1. Effect of different plant extracts on the growth of A. flavus at concentrations of 100, 300, and 600 mg/ml. S = Standard (Fluconazole); C = Control (Blank disc).

Table 4. Effect of plant extracts and fungicide on the growth of *A. flavus*.

Plants	Mean zone of Inhibition (mm) ± SE at				CV(%)
	600 mg/ml	300 mg/ml	100 mg/ml	Standard (Fluconazole) (150 mg/ml)	
Azadirachta indica	19.83 ± 0.31a	14.67 ± 0.33c	10.67 ± 0.42d	15.67 ± 0.00b	4.99
Piper betle	14.50 ± 0.76a	12.50± 0.22b	8.67 ± 0.33c	15.67 ± 0.00a	4.23
Centella asiatica	11.33 ± 0.33b	9.33 ± 0.49c	9.17 ± 0.31c	15.67 ± 0.00a	4.22
Ocimum	9.17 ± 0.31b	8.17 ± 0.31c	0.00 ± 0.00	15.67 ± 0.00a	4.45
Mentha sapicata	0.00 ± 0.00b	0.00 ± 0.00b	0.00 ± 0.00b	15.67 ± 0.00a	4.45

The numbers represent the means of six replications. One-way ANOVA was used for analysis, and means were separated using the LSD test. Numbers followed by the same letter in the same row are not significantly different ($p < 0.05$).

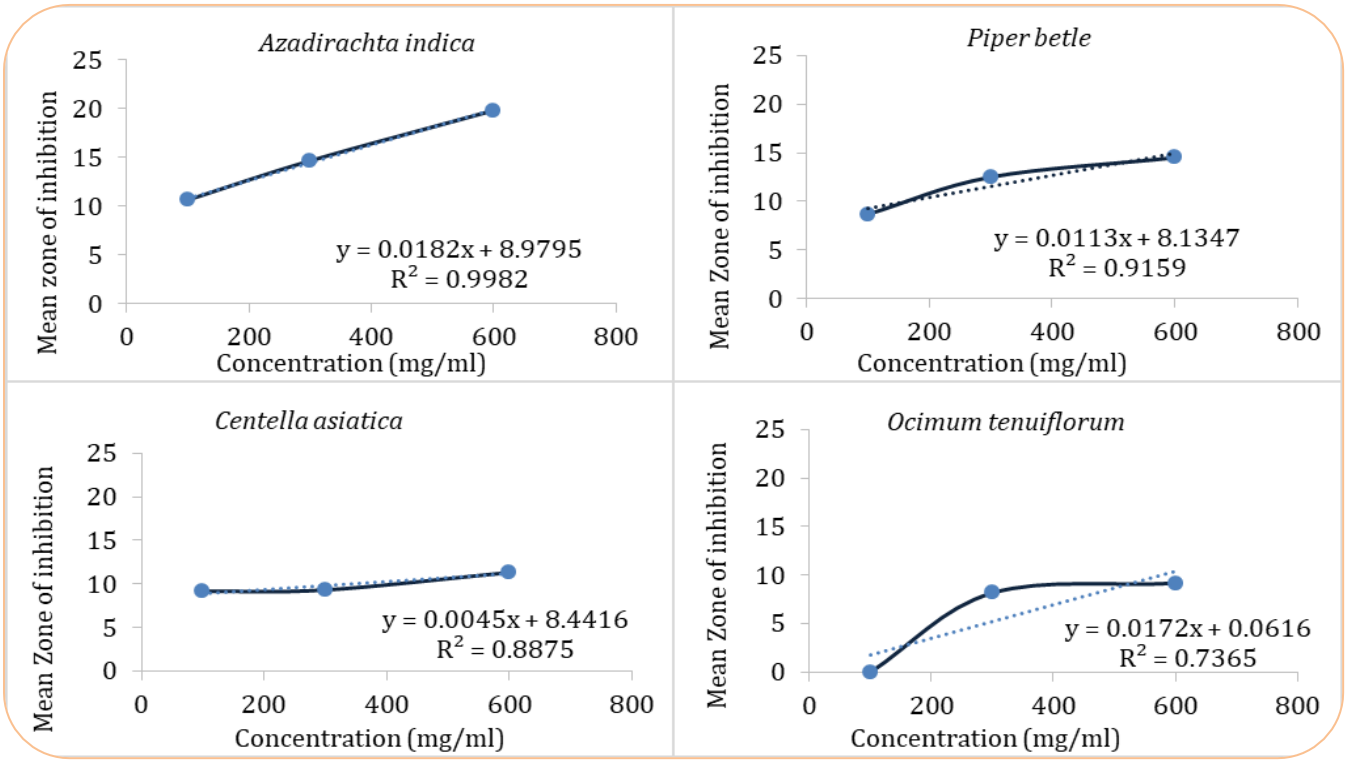

Figure 2. The functional relationship between botanical concentration and inhibition frequency.

Determination of MIC

The susceptibility of A. flavus to A. indica, P. betle, C. asiatica, and O. tenuiflorum was assessed on the basis of their MIC values. To evaluate the antifungal activity of these extracts, MIC values were determined for each extract at different concentrations (350, 150, 75, 18.75, 9.375, and 4.687 mg/ml), with Fluconazole serving as the reference antifungal agent (Table 5).

The methanolic extract of *A. indica* exhibited potent antifungal activity, with a MIC value of 9.375 mg/ml, requiring the lowest concentration compared to other plant extracts. *P. betle* and *C. asiatica* demonstrated moderate antifungal efficacy, with MIC values of 18.75 mg/ml, 37.5 mg/ml, 75 mg/ml, 150 mg/ml, and 300 mg/ml. Notably, *O. tenuiflorum* exhibited inhibitory effects at concentrations of 150 mg/ml and 300 mg/ml, indicating its effectiveness against *A. flavus*.

In comparison, the synthetic antifungal agent Fluconazole displayed a MIC value of 4.68 mg/ml, exhibiting inhibitory potency similar to *A. indica* extract. The presence of antifungal activity in the extracts was indicated by a blue color, signifying no visible fungal growth. MIC values for each extract against *A. flavus* were determined separately. The variation in MIC values highlights the diverse efficacy of these plant extracts against *A. flavus*. These findings suggest that MIC values are concentration-dependent.

Table 5. Various concentrations used to determine the minimum inhibitory concentration.

Plant extract	Concentration (mg/ml)						
	4.687	9.375	18.75	37.5	75	150	300
Azadirachta indica	+	-	-	-	-	-	-
Piper betle	+	+	-	-	-	-	-
Centella asiatica	+	+	-	-	-	-	-
Ocimum tenuiflorum	+	+	+	+	+	-	-
Mentha spicata	+	+	+	+	+	+	+
Standard (Fluconazole)	-	-	-	-	-	-	(not used)

Grow (+), Not Grow (-).

Genetic diversity analysis through RAPD method

DNA fingerprint analysis

Genetic variation among the 12 isolates of *A. flavus* was analyzed using 12 RAPD primers, which amplified a maximum of 50 DNA fragments. All tested primers exhibited 100% polymorphism. Among them, OPM-10 generated the highest number of amplified fragments and produced distinct amplification patterns.

According to Muller et al. (2005), DNA fragments present in at least one isolate and up to a maximum of 90% of isolates (i.e., in 5-90% of cases) were considered polymorphic, while fragments detected in at least 11 isolates (95% occurrence) were classified as monomorphic. Based on this criterion, all amplified fragments were found to be 100% polymorphic (Table 6). The PCR-amplified products using RAPD primers are presented in Figure 3.

Cluster analysis and evolutionary relationships among the isolates

The dendrogram was constructed based on data generated from the amplification of 12 isolates using 12 primers, revealing three distinct clusters (Figure 4a). Cluster I consists of three isolates, while Cluster II contains nine isolates and is further subdivided into two sub-clusters.

The Neighbour-Joining (NJ) tree illustrates the evolutionary relationships among the isolates. To construct the NJ tree, AS-01 was used as the common emerging point (ancestor) (Figure 4b). The analysis revealed two distinct groups: Cluster I, which contains only three isolates, and Cluster II, which includes the remaining isolates.

Table 6. Primers used for RAPD analysis of 12 isolates of *A. flavus* and the number of amplified DNA fragment.

Sr. No.	Primer Code	Maximum number of amplified bands	Number of Polymorphic bands	Number of Monomorphic bands	Polymorphism (%)
1	OPA-18	03	03	0.0	100
2	OPF-04	05	05	00	100
3	OPF-05	02	02	00	100
4	OPM-04	05	05	00	100
5	OPM-10	07	07	00	100
6	OPM-12	04	04	00	100
7	OPP-04	04	04	00	100
8	OPW-02	03	03	00	100
9	OPW-05	05	05	00	100
10	UBC-25	03	03	00	100
11	UBC-155	03	03	00	100
12	UBC-173	06	06	00	100
	Total	50	50	00	100

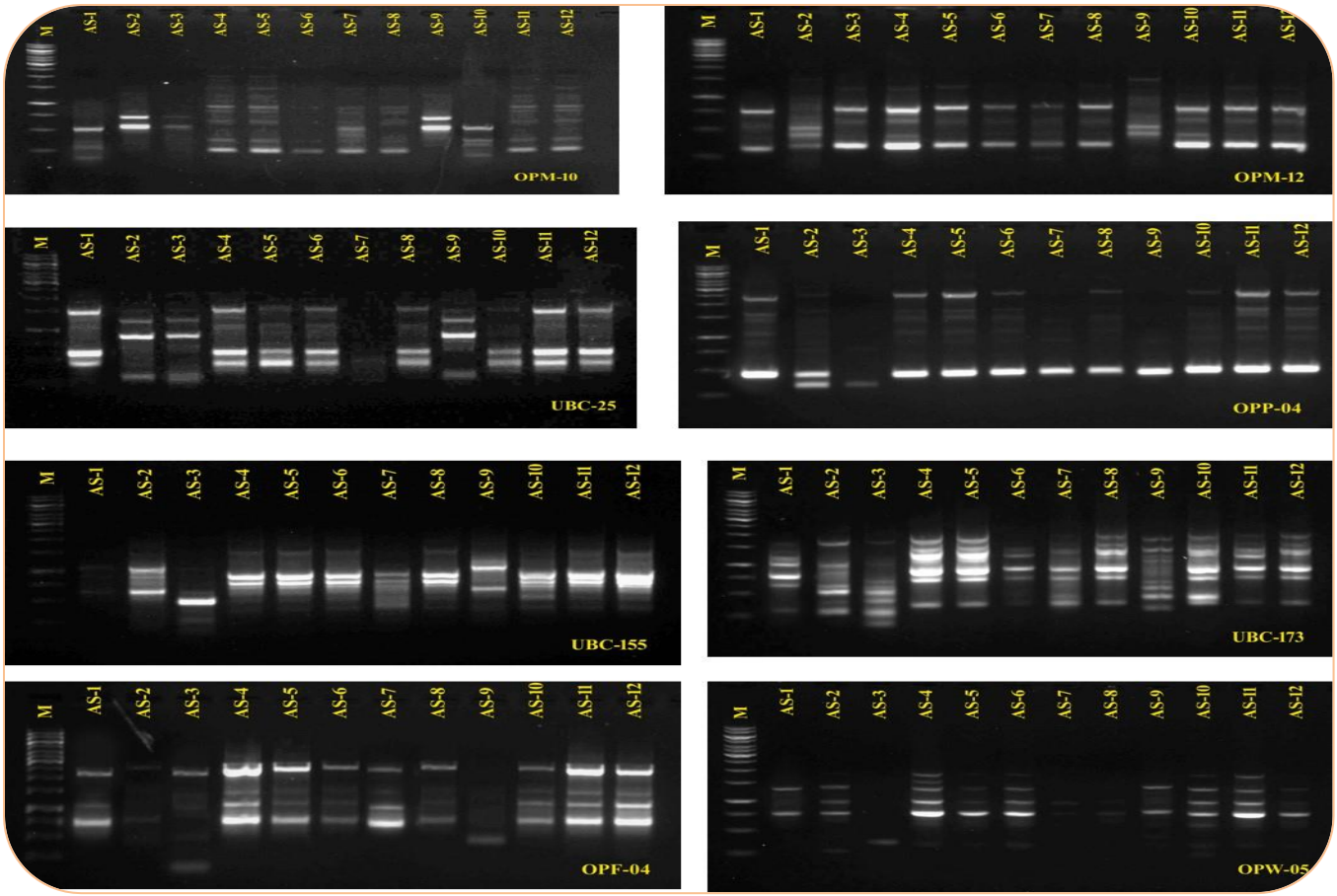

Figure 3. DNA fragment profiles of 12 isolates of *A. flavus* generated by using RAPD primers and an M- Kilo base molecular weight ladder.

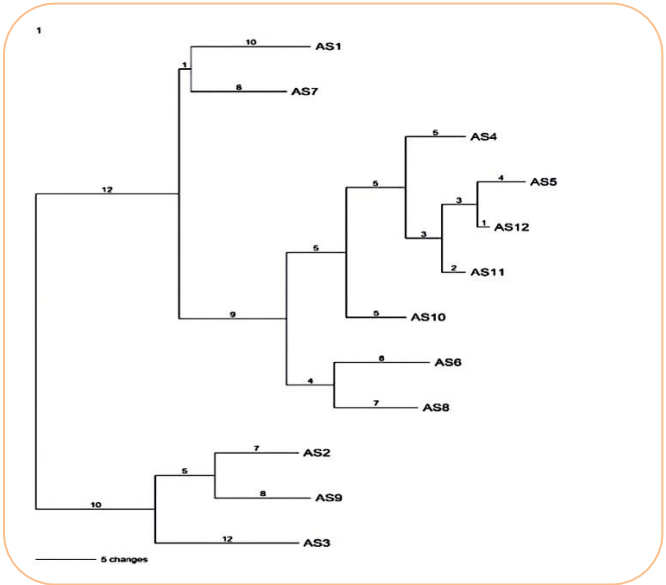

Figure 4(a). Grouping of isolates based on DNA fingerprinting and evolutionary relationships among *A. flavus* isolates.

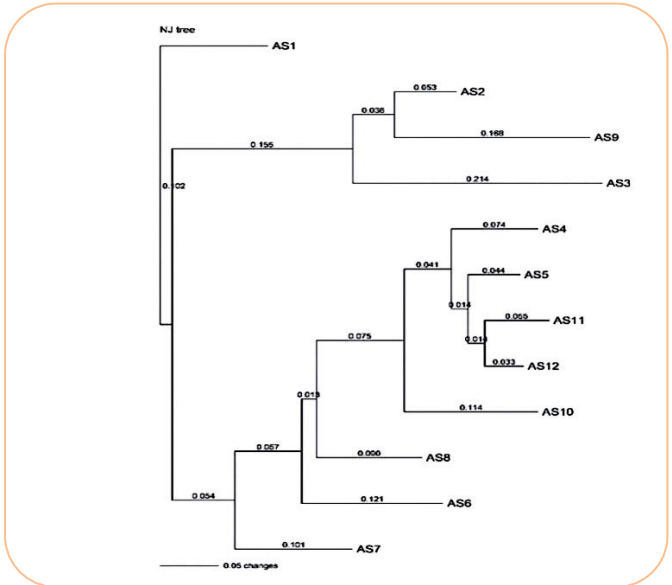

Figure 4(b). Grouping of isolates based on DNA fingerprinting and evolutionary relationships among *A. flavus* isolates.

Genetic distances among 12 isolates of *A. flavus* were evaluated based on DNA polymorphism (Table 7). The highest genetic similarity (99.2%) was observed between AS11 and AS12. In contrast, the greatest genetic distance (73%) was found between AS3 and AS4, indicating significant genetic divergence between these isolates. Most pairwise genetic distances ranged from 0.07 to 0.73, reflecting varying levels of genetic diversity among the isolates. Some isolates, such as AS5 and AS6, exhibited moderate genetic distances from other isolates.

Table 7. Pair-wise genetic distance of 12 isolates of *A. flavus* based on DNA polymorphism.

Sample	AS1	AS2	AS3	AS4	AS5	AS6	AS7	AS8	AS9	AS10	AS11	AS12
AS1	-											
AS2	0.39											
AS3	0.43	0.35										
AS4	0.45	0.55	0.73									
AS5	0.38	0.51	0.72	0.23								
AS6	0.32	0.48	0.57	0.33	0.32							
AS7	0.26	0.39	0.48	0.43	0.32	0.29						
AS8	0.33	0.47	0.61	0.26	0.25	0.22	0.25					
AS9	0.50	0.22	0.36	0.69	0.65	0.56	0.56	0.55				
AS10	0.38	0.52	0.60	0.22	0.23	0.32	0.29	0.31	0.61			
AS11	0.42	0.53	0.68	0.15	0.13	0.28	0.37	0.32	0.72	0.22		
AS12	0.39	0.52	0.73	0.15	0.07	0.34	0.36	0.26	0.69	0.22	0.08	-

DISCUSSION

Numerous studies have highlighted the role of the opportunistic fungus *A. flavus* as a significant contaminant of chili. Its presence not only compromises chili quality but also raises food safety concerns, emphasizing the need for effective management strategies to minimize contamination risks (Jannat et al., 2022). The current study aimed to evaluate the efficacy of selected botanical extracts, in comparison to the synthetic fungicide Fluconazole, against aflatoxin-producing *A. flavus*. Moreover, the study analyzed the genetic diversity of *A. flavus* isolates from chilies using RAPD markers. The isolates were identified as pathogenic based on the criteria established by Okayo et al. (2020).

To prevent aflatoxin contamination, plant extracts derived from various botanical sources serve as potent reservoirs of bioactive compounds with remarkable antimicrobial and antifungal properties (Bharathi et al., 2014). Among these, *A. indica* exhibited the most significant inhibitory effects, with a zone of inhibition measuring 19.83 ± 0.31 mm at 600 mg/mL, outperforming Fluconazole (15.67 ± 0.00 mm at 150 mg/ml). This finding aligns with previous studies highlighting the strong antifungal properties of *A. indica*, attributed to bioactive compounds such as nimbidin and azadirachtin (Shrivastava and Swarnkar, 2014).

In this study, the organic extract of *C. asiatica* demonstrated significant antifungal activity against *A. flavus*, with inhibition zones ranging from 9.17 mm to 11.33 mm. These results are consistent with those of Dash et al. (2011), who reported inhibition zones of 11-14 mm for the organic extract. However, the aqueous extract showed lower efficacy, with an inhibition zone of 11 mm in the same study. *O. tenuiflorum* exhibited inconsistent antifungal activity, while *M. spicata* had negligible effects, indicating variability in their antifungal potential.

The significant antimicrobial activity of *A. indica*, even at lower concentrations (100 mg/ml), and the moderate effectiveness of *P. betle* and *C. asiatica* at the same concentration suggest their potential as natural antifungal agents. These findings emphasize the importance of exploring plant-based antifungal compounds as sustainable alternatives to synthetic agents like Fluconazole.

It was observed that plant extracts exhibiting strong antifungal activity had the lowest MIC values against *A. flavus*. The methanolic extract of *A. indica* demonstrated potent antifungal activity in this study, with an MIC value of 9.375 mg/ml against *A. flavus*, requiring the lowest concentration among all tested plant extracts. This finding highlights the potential of *A. indica* as an effective natural antifungal agent.

In comparison, a study by Margaret et al. (2013) reported a higher MIC value of 5000 µg/ml (0.5 g/dl) for the ethanol extract of *A. indica* leaves against *A. flavus*. The variation in MIC values between the two studies may be attributed to differences in extraction solvents, with methanol potentially yielding more active antifungal compounds than ethanol.

In our study, the methanolic extract of *P. betle* exhibited moderate antifungal activity against *A. flavus*, with MIC values ranging from 18.75 mg/ml to 300 mg/ml, while *C. asiatica* showed inhibitory effects at 150 mg/ml and 300 mg/ml. Comparatively, Basak and Guha (2017) reported that the essential oil of *P. betle*, formulated into a microemulsion, effectively inhibited *A. flavus* growth in tomato paste. Furthermore, an ethanolic extract of *C. asiatica* demonstrated significant antifungal activity, with a 24.4 mm inhibition zone against *A. flavus* at 100% concentration, although specific MIC values were not provided.

Variations in antifungal efficacy may result from differences in extraction methods, solvents, or formulations, emphasizing the need for standardized experimental protocols. Furthermore, differences in active compounds, such as flavonoids (apigenin, vicenin, and orientin), phenolic compounds (cirsilineol, circimaritin, isothymusin, and eugenol), and ursolic acid, may contribute to the observed discrepancies.

In the present study, twelve isolates of *A. flavus* underwent RAPD analysis to assess genetic diversity. Twelve primers generated a total of 50 bands, with no monomorphic bands observed (Table 6). On average, each primer produced 4.16 bands, all of which were polymorphic. The number of amplified fragments per primer ranged from 2 to 7, with fragment sizes varying from 250 to 3500 bp. Differences in the number of amplified bands were influenced by various factors, such as primer sequence and a lower number of annealing loci in the genome. Some isolates were distinguished by unique bands, either present or absent. The presence of a unique band in a specific isolate was considered a positive marker, whereas the absence of a common band was regarded as a negative marker.

Among the primers, OPM-10 and UBC-173 generated the highest number of bands, with molecular sizes ranging from 260 to 3500 bp. The UBC-173 primer specifically distinguished isolates AS4 and AS5 by producing a unique 2000 bp band (Figure 3). The highest genetic similarity (99.2%) was observed between isolates AS11 and AS12, whereas the greatest genetic diversity (73%) was found between isolates AS3 and AS4 (Table 7).

The genetic similarity between AS3 and AS4 indicates moderate genetic diversity within the population. This moderate variation suggests that while the isolates share genetic similarities, there is enough diversity to influence traits such as aflatoxin production. In contrast, AS11 and AS12 exhibit nearly identical aflatoxin-producing capacities. A recent study by Mesterházy (2024) highlighted that *A. flavus* populations with high genetic similarity often maintain stable toxin production but may exhibit subtle differences in response to external stressors, such as host resistance or climate variability.

Cluster analysis revealed two distinct groups, with a maximum genetic distance of 73% among *A. flavus* isolates from Bangladesh, indicating that plant-pathogenic fungi commonly rely on genetic recombination to enhance gene flow (McDermott and McDonald, 1993; Burdon and Silk, 1997). Although RAPD-PCR demonstrated significant discriminatory power among the examined isolates, the level of diversification detected by rep-PCR was less pronounced. Varga et al. (2005) used RAPD-PCR and observed notable variability among *A. terreus* isolates. Similarly, our RAPD data revealed significant interstrain variability, particularly at a similarity value of ≥ 99%. This finding aligns with previous studies showing that *A. flavus* exhibits substantial genetic diversity.

The use of molecular marker technology for the rapid detection and characterization of specific fungal species is gaining increasing attention, as it plays a crucial role in effective disease management and in breeding plants resistant to virulent isolates of *Aspergillus* spp., which are prevalent across various regions worldwide. The findings of this study indicate that *A. flavus* associated with chili crops across diverse agro-climatic regions of Bangladesh exhibits considerable genetic diversity, which may help predict its aflatoxin production potential. This study also highlights the importance of considering different medicinal plant extracts and their concentrations when exploring natural alternatives to synthetic antifungal agents.

CONCLUSION

The present study emphasized aflatoxin contamination and the associated health risks. Molecular identification confirmed *Aspergillus flavus* with 99.08% similarity to the GenBank reference OP480002.1. Medicinal plants

demonstrated promising antifungal properties against *A. flavus*. *Azadirachta indica* surpassed Fluconazole in effectiveness, while *Piper betle* exhibited comparable inhibition at 600 mg/ml. Fluconazole had the lowest MIC value, whereas *Centella asiatica* and *Ocimum tenuiflorum* showed moderate inhibition, and *Mentha spicata* exhibited none. Future research should focus on isolating bioactive compounds from these plants for sustainable aflatoxin mitigation in crops. RAPD analysis revealed substantial genetic diversity among the isolates, with 50 polymorphic bands ranging from 250 to 3500 bp and unique variations, such as the 2000 bp band in isolates 4 and 5. The highest genetic similarity (99.2%) was observed between AS11 and AS12, while the greatest genetic distance (73%) was found between AS3 and AS4. These findings indicate that genetic diversity plays a crucial role in identifying aflatoxigenic genotypes, aiding in the development of effective biocontrol strategies.

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AUTHORS' CONTRIBUTIONS

SS and NJ designed the study, developed the experimental framework, and conducted the experiments; SMAAM, SAKU, and MMR collected, organized, and analyzed the data; SS and NJ wrote the manuscript and contributed to its revision and proofreading.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

REFERENCES

Adebiyi, J.A., Kayitesi, E., Adebo, O.A., Changwa, R., Njobeh, P.B., 2019. Food fermentation and mycotoxin detoxification: An African perspective. Food Control 106, 106731.

Basak, S., Guha, P., 2017. Betel leaf (*Piper betle* L.) essential oil microemulsion: Characterization and antifungal activity on growth, and apparent lag time of *Aspergillus flavus* in tomato paste. LWT 75, 616-623.

Behiry, S.I., Hamad, N.A., Alotibi, F.O., Al-Askar, A.A., 2022. Antifungal and antiaflatoxigenic activities of different plant extracts against *Aspergillus flavus*. Sustainability 14(19), 12908.

Bharathi, T., Kolanjinathan, K., Saranraj, P., 2014. Antimicrobial activity of solvent extracts of *Ocimum tenuiflorum*, *Azadirachta indica,* and *Phyllanthus amarus* against clinical Pathogens. Global Journal of Pharmacology 8(3), 294-305.

Burdon, J., Silk, J., 1997. Sources and patterns of diversity in plant-pathogenic fungi. Phytopathology 87(7), 664-669.

Chongo, G., Gossen, B.D., Buchwaldt, L., Adhikari, T., Rimmer, S., 2004. Genetic diversity of *Ascochyta rabiei* in Canada. Plant disease 88(1), 4-10.

Chukwudi, H.C., and Ezeabara, C.A., 2018. Phytochemical screening and in vitro antimicrobial activities of Mimosa invisa Mart. leaves and stems. *Bioscience Horizons: The International Journal of Student Research*, *11*, hzy019.

Di Ciaccio, L., Catalano, A., López, P., Salvat, A., 2020. *In vitro* antifungal activity of *Peltophorum dubium* (Spreng.) taub. extracts against *Aspergillus flavus*. Plants 9(4), 438.

FAOSTAT., 2017. FAO, available online: http://www. fao. org/faostat/en/# data. QC (accessed on January 2018).

Hossain, M.N., Talukder, A., Afroze, F., Rahim, M.M., Begum, S., Haque, M.Z., Ahmed, M. M., 2018. Identification of aflatoxigenic fungi and detection of their aflatoxin in red chili (*Capsicum annuum*) samples using direct cultural method and HPLC. Advances in Microbiology 8(1), 42-53.

Hudzicki, J., 2009. Kirby-Bauer disk diffusion susceptibility test protocol. American Society for Microbiology 15, 55-63.

Jannat, M., Masud, M., Nusrat, M., Bashar, S., Mita, M.M., Hossain, M.I., and Islam, M.R., 2022. Aflatoxins and fumonisins contamination of maize in Bangladesh: an emerging threat for safe food and food security. maize genetic resources: Breeding Strategies and Recent Advances 18-69.

Kavitha, K., Vijaya, N., Krishnaveni, A., Arthanareeswari, M., Rajendran, S., Al-Hashem, A., Subramania, A., 2020. Nanomaterials for antifungal applications. In Nanotoxicity. Elsevier 385-398.

Kennedy, Z.J., Paranitharan, V., Karthikeyan, S., 2022. Mycotic contamination and aflatoxin potential of molds in *Capsicum annum* (chili), and chili powder commercialized in south Indian markets. Toxicon 210, 109-114.

Khan, M.A., Asghar, M.A., Iqbal, J., Ahmed, A.,

Shamsuddin, Z.A., 2014. Aflatoxins contamination and prevention in red chillies (*Capsicum annuum* L.) in Pakistan. Food Additives & Contaminants: Part B 7(1), 1-6.

Maida, C.M., Milici, M.E., Trovato, L., Oliveri, S., Amodio, E., Spreghini, E., and Barchiesi, F., 2008. Evaluation of the disk diffusion method compared to the microdilution method in susceptibility testing of anidulafungin against filamentous fungi. Journal of Clinical Microbiology 46(12), 4071-4074.

Makhuvele, R., Naidu, K., Gbashi, S., Thipe, V.C., Adebo, O.A., Njobeh, P.B 2020. The use of plant extracts and their phytochemicals for control of toxigenic fungi and mycotoxins. Heliyon 6(10).

Margaret, A., Yolanda, H., 2013. Antifungal activity of neem leaf ethanol extract on Aspergillus flavus. Universa Medicina 32(2), 80-85.

McDonald, B.A., McDermott, J.M., 1993. Population genetics of plant pathogenic fungi. Bioscience 43(5), 311-319.

Mesterhazy, A., 2024. Food safety aspects of breeding maize to multi-resistance against the major *(Fusarium graminearum*, *F. verticillioides*, *Aspergillus flavus*) and minor toxigenic fungi (*Fusarium* spp.) as well as to toxin accumulation, trends, and solutions-a review. Journal of Fungi 10(1), 40.

Müller, S., Schlöder, F., Stutzki, J., Winnewisser, G., 2005. The cologne database for molecular spectroscopy, CDMS: a useful tool for astronomers and spectroscopists. Journal of Molecular Structure 742(1-3), 215-227.

Njoki, L.M., Okoth, S.A., Wachira, P.M., 2017. Effects of medicinal plant extracts and photosensitization on aflatoxin producing *Aspergillus flavus* (Raper and Fennell). International Journal of Microbiology 2017, 1-9.

Okayo, R.O., Andika, D.O., Dida, M.M., K'Otuto, G.O., Gichimu, B.M., 2020. Morphological and molecular characterization of toxigenic *Aspergillus flavus* from groundnut kernels in Kenya. International Journal of Microbiology 2020, 1-10.

Prakash, B., Kumar, A., Singh, P.P., Songachan, L.S., 2020. Antimicrobial and antioxidant properties of phytochemicals: Current status and future perspective. Functional and Preservative Properties Of Phytochemicals 1-45.

Saitou, N., Nei, M., 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Molecular Biology and Evolution 4(4), 406-425.

Shahi, S.K., Patra, M., Shukla, A.C. Dikshit, A., 2003. Use of essential oil as botanical-pesticide against post harvest spoilage in *Malus pumilo* fruit. BioControl 48, 223-232.

Shrivastava, D.K., Swarnkar, K., 2014. Antifungal activity of leaf extract of neem (*Azadirachta indica* Linn). International Journal of Current Microbiology and Applied Sciences 3(5), 305-308.

Singh, P., Cotty, P.J., 2019. Characterization of Aspergilli from dried red chilies (*Capsicum* spp.): insights into the etiology of aflatoxin contamination. International Journal of Food Microbiology 289, 145-153.

Sneath, P.H., Sokal, R.R., 1973. Numerical Taxonomy: The Principles and Practice of Numerical Classification. WF Freeman & Co., San Francisco, 573 p.

Szabo, B., Toth, B., Toth Toldine, E., Varga, M., Kovacs, N., Varga, J., Mesterházy, A., 2018. A new concept to secure food safety standards against *Fusarium* species and *Aspergillus flavus* and their toxins in maize. Toxins 10(9), 372.

Varga, J., Tóth, B., Kocsubé, S., Farkas, B., Szakács, G., Téren, J., & Kozakiewicz, Z., 2005. Evolutionary relationships among *Aspergillus terreus* isolates and their relatives. Antonie Van Leeuwenhoek, 88, 141-150.