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Detection And Quantification of Mycotoxins in Turmeric, Cloves, And Nutmeg

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Abstract- ***Spices such as turmeric (Curcuma longa), cloves (Syzygium aromaticum), and nutmeg (Myristica fragrans) are widely used for culinary and medicinal purposes but are susceptible to fungal contamination and mycotoxin accumulation, posing potential health risks. This study evaluated the physicochemical characteristics, occurrence and co-occurrence of mycotoxins, structural confirmation, and the impact of storage conditions on toxin accumulation in selected spices. Turmeric, cloves, and nutmeg samples were obtained from Big Tree Market in Rumuolumeni, Port Harcourt, cleaned, ground into powder, and analyzed in triplicate. Moisture content was determined using the oven-drying method, water activity using a water activity meter, and pH using a calibrated digital pH meter. Mycotoxins (aflatoxins B_1, B_2, G_1, G_2 and ochratoxin A) were extracted using methanol–water and acetonitrile–water solvents and analyzed using ELISA for screening and LC-MS/MS for confirmation and quantification. Structural characterization was performed based on molecular weight, retention time, and fragmentation ions, while storage experiments were conducted at 25–45 °C and 50–90% relative humidity for 0–3 months. Results showed that turmeric had moisture content of 8.5%, water activity of 0.52 and pH 5.5, cloves recorded 10.0% moisture, aw 0.58 and pH 6.2, while nutmeg had 7.2% moisture, aw 0.48 and pH 6.5. Aflatoxin B_1 levels were 5.0 µg/kg in turmeric, 6.3 µg/kg in cloves, and 3.9 µg/kg in nutmeg, while ochratoxin A levels were 2.0 µg/kg, 2.8 µg/kg, and 1.4 µg/kg respectively. Other aflatoxins (B_2, G_1, G_2) occurred at trace levels (0.3–1.6 µg/kg). Storage experiments showed that aflatoxin B_1 in turmeric increased from 4.8 µg/kg initially to 6.5 µg/kg after 3 months at 25 °C and 50% RH and reached 8.2 µg/kg at 35 °C and 70% RH, while ochratoxin A increased to 10.5 µg/kg at 45 °C and 90% RH. Similar increases were observed in cloves and nutmeg. The study concludes that although initial mycotoxin levels were within safe limits, high temperature, humidity, and prolonged storage significantly promote toxin accumulation. Proper storage conditions and regular monitoring of spices are therefore recommended to ensure food safety and protect public health.***

Index Terms- ***Detection, Quantification, Mycotoxins, Turmeric, Cloves, And Nutmeg***

I. INTRODUCTION

Spices play a crucial role in culinary practices, food preservation, and the pharmaceutical industry due to their flavor, aroma, and bioactive compounds. Among widely consumed spices, turmeric, cloves, and nutmeg are particularly valued for their medicinal and antioxidant properties. However, these spices are highly susceptible to fungal contamination during pre-harvest, post-harvest, and storage, which can lead to the production of mycotoxins, secondary metabolites produced by toxigenic fungi that pose serious health risks to humans and animals. Mycotoxins such as aflatoxins, ochratoxins, and fumonisins are recognized for their carcinogenic, nephrotoxic, immunosuppressive, and hepatotoxic effects. The contamination of spices with mycotoxins has become a global concern because these products are often stored for extended periods under suboptimal conditions of temperature and humidity, which favor fungal growth and toxin accumulation. Moreover, spices are frequently imported and traded across regions, raising significant food safety and public health concerns, as contamination may go unnoticed due to the low moisture content of these products. Effective detection and quantification of mycotoxins are essential for ensuring food safety. Traditional analytical methods such as enzyme-linked immunosorbent assay (ELISA) provide rapid and cost-effective screening for mycotoxins, while liquid chromatography-tandem mass spectrometry (LC-MS/MS) offers high sensitivity and specificity for

precise quantification and structural characterization. Combining these techniques allows comprehensive assessment of mycotoxin prevalence, concentration, and co-occurrence, which is critical for risk assessment and regulatory compliance (Alshammari et al., 2024).

Demirhan and Demirhan (2023) revealed the presence of aflatoxin B1, B2, G1 and G2 in several turmeric samples, with some concentrations approaching or exceeding recommended safety limits, indicating the need for routine monitoring of spices in commercial market, Juma et al. (2024) showed that a large proportion of turmeric samples contained detectable levels of aflatoxin B1, although most values were within permissible international safety limits, Haq et al. (2024) revealed detectable levels of aflatoxin B1 and ochratoxin A in several clove samples, indicating contamination associated with fungal growth during storage, Stroka et al. (2019) demonstrated that the analytical method was highly sensitive and reproducible across laboratories, confirming its suitability for routine monitoring of mycotoxins in spices, Manda et al. (2022) indicated that aflatoxin B1 and ochratoxin A were the most frequently detected toxins and that some spice samples contained multiple toxins simultaneously, Kumar and Singh (2021) showed that several turmeric samples contained detectable levels of aflatoxin B1, though most values remained within acceptable limits, Rahman et al. (2022) revealed that Aspergillus flavus and Aspergillus parasiticus were the main fungal species responsible for aflatoxin production in clove samples, Adeyeye and Oyedeji (2023) showed measurable levels of aflatoxin B1 and ochratoxin A in several nutmeg samples, indicating that improper storage contribute to contamination, Alshammari et al. (2024) revealed the presence of mycotoxin-producing fungi capable of generating aflatoxins and ochratoxin A in several spice samples, Demirel et al. (2021) showed that aflatoxin B1 and ochratoxin A were frequently detected in spice samples although their concentrations varied depending on storage conditions, Njoroge et al. (2022) indicated that turmeric and nutmeg occasionally exceeded recommended aflatoxin limits, highlighting potential health risks, Patel et al. (2020) revealed detectable concentrations of ochratoxin A in several nutmeg samples, Singh and Sharma (2021) showed that several samples contained aflatoxin B1 although most were below regulatory safety limits, Okeke et al. (2022) revealed detectable levels of aflatoxin B1 in some spice samples, suggesting the need for improved food safety monitoring, Hassan et al. (2023) indicated that warm and humid storage conditions significantly increased the presence of aflatoxin B1 and ochratoxin A in turmeric and nutmeg samples, Ghosh et al. (2021) revealed that turmeric samples showed higher contamination levels compared to cloves. López et al. (2022) confirmed the presence of ochratoxin A in a number of spice samples, Chen et al. (2023) showed that ELISA provided rapid detection while LC-MS/MS offered more precise quantification of aflatoxins and ochratoxin A, Akinola et al. (2022) demonstrated that higher fungal counts were associated with increased aflatoxin levels in turmeric and nutmeg samples, and Silva et al. (2023) showed that although most spice samples were within acceptable limits, a few turmeric and nutmeg samples exceeded recommended aflatoxin levels, emphasizing the importance of regular monitoring and improved storage practices.

II. AIM AND OBJECTIVES OF THE STUDY

The aim of this research is to determine the presence and concentration of harmful mycotoxins in selected spice samples (turmeric, cloves, and nutmeg) while the objectives were to:

i. characterize (moisture content, water activity, and pH) turmeric, cloves, and nutmeg;
ii. detect and quantify mycotoxins produced in turmeric, cloves, and nutmeg using LC-MS/MS and ELISA.
iii. evaluate the co-occurrence patterns of multiple mycotoxins in turmeric, cloves, and nutmeg;
iv. structurally and chemically characterize the detected mycotoxins using LC-MS/MS and ELISA;
v. assess the impact of storage conditions (temperature, humidity, and duration) on mycotoxin accumulation in turmeric, cloves, and nutmeg.

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III. MATERIALS AND METHODS

3.1 Characterization of Spices (turmeric, cloves, and nutmeg)

3.1.1 Sample Collection and Preparation

Turmeric, cloves, and nutmeg samples were obtained from big tree markets in Rumuolumeni Port-Harcourt and taken to the laboratory for analysis. The samples were cleaned to remove foreign materials such as stones, dust, and plant debris. Each spice sample was then ground into fine powder using a clean laboratory grinder. The powdered samples were stored in sterile airtight containers to prevent contamination and moisture absorption prior to analysis. All analyses were carried out in triplicate to ensure accuracy and reliability of the results.

3.1.2 Determination of Moisture Content

The moisture content of turmeric, cloves, and nutmeg was determined using the oven-drying method. Approximately 5 g of each powdered spice sample was weighed using a digital analytical balance and placed in a pre-weighed moisture dish or crucible. The samples were dried in a hot air oven at 105°C for about 3–4 hours until a constant weight was obtained. After drying, the samples were transferred into a desiccator and allowed to cool for about 30 minutes to prevent moisture absorption from the atmosphere. The cooled samples were reweighed using the analytical balance. The moisture content was calculated as the percentage loss in weight during drying using the formula:

$$\text{Moisture Content (\%)} = \frac{\text{Initial weight of sample} - \text{Final weight of dried sample}}{\text{Initial weight of sample}} \times 100$$

3.1.3 Determination of Water Activity (aw)

Water activity of turmeric, cloves, and nutmeg samples was measured using a water activity meter. About 2–3 g of each powdered spice sample was placed in the sample cup of the water activity meter. The sample chamber was sealed, and the sample was allowed to equilibrate within the instrument at room temperature. The instrument measured the equilibrium relative humidity of the sample and automatically converted it to water activity (aw). The value was displayed on the digital screen of the meter.

3.1.4 Determination of pH

The pH of the spice samples was determined using a digital pH meter. Approximately 10 g of each powdered sample was mixed with 90 ml of distilled water in a sterile beaker to prepare a suspension. The mixture was stirred thoroughly using a glass rod and allowed to stand for about 30 minutes to ensure proper extraction of soluble components. The pH meter was calibrated using standard buffer solutions of pH 4.0 and 7.0 before measurement. The electrode of the pH meter was immersed into the spice suspension, and the pH value was recorded once the reading stabilized. The electrode was rinsed with distilled water between measurements to avoid cross-contamination.

3.2 Detection and Quantification of Mycotoxins Using ELISA/ LC-MS/MS

Approximately 5 g of each powdered spice sample was weighed and placed in a clean extraction flask. About 25 ml of 70% methanol (methanol–distilled water, 70:30 v/v) was added as the extraction solvent. The mixture was shaken for about 30 minutes using a mechanical shaker to allow the mycotoxins present in the samples to dissolve into the solvent. The mixture was filtered using Whatman No. 1 filter paper to obtain a clear extract. A portion of the filtrate was diluted with distilled water according to the instructions provided with the ELISA kit. ELISA test kits containing enzyme conjugate, specific antibodies, washing buffer, chromogenic substrate (Tetramethylbenzidine – TMB), and stop solution (1 N sulfuric acid, H_2SO_4) were used for the analysis. Standard solutions and prepared sample extracts were pipetted into the antibody-coated wells of the ELISA microplate. The enzyme conjugate reagent was added to each well, followed by incubation for the specified time to allow antigen–antibody binding reactions to occur. After incubation, the wells were washed several times with phosphate-buffered saline (PBS) washing buffer to remove unbound substances. A TMB substrate solution was then added to each well and allowed to react to produce a blue coloration. The reaction was stopped by adding 1 N sulfuric acid (H_2SO_4) which changed the color to yellow. The absorbance was measured using an ELISA

microplate reader at 450 nm wavelength. The concentration of mycotoxins in the samples was determined by comparing the absorbance values obtained from the samples with those from the standard calibration curve.

Approximately 5 g of each powdered spice sample was weighed into a centrifuge tube. About 20 ml of acetonitrile–water solution (80:20 v/v) was added as the extraction solvent. In addition, sodium chloride (NaCl) and magnesium sulfate ($MgSO_4$) were added to facilitate phase separation during extraction. The mixture was vigorously shaken for about 30 minutes to ensure efficient extraction of the mycotoxins. The mixture was centrifuged at 4000 rpm for about 10 minutes to separate the solid particles from the extract. The supernatant was carefully collected and purified using an immunoaffinity column (IAC) or solid-phase extraction (SPE) column containing C18 sorbent to remove pigments, oils, and other interfering substances present in the spice matrix. The purified extract was filtered through a 0.22 µm polytetrafluoroethylene (PTFE) membrane filter. A measured volume of the filtrate was injected into the LC-MS/MS system. The liquid chromatography separation was carried out using a C18 reversed-phase column, with mobile phases consisting of water with 0.1% formic acid (Solvent A) and acetonitrile with 0.1% formic acid (Solvent B). The mass spectrometer operated in electrospray ionization (ESI) mode and detected mycotoxins based on their mass-to-charge ratios (m/z). Calibration standards prepared from aflatoxin B_1, aflatoxin B_2, aflatoxin G_1, aflatoxin G_2, and ochratoxin A standard solutions were analyzed under the same conditions to generate calibration curves. The concentration of each mycotoxin in the spice samples was calculated by comparing the chromatographic peak areas of the samples with those of the calibration standards.

3.3 Co-occurrence patterns of multiple mycotoxins in turmeric, cloves, and nutmeg

Approximately 5 g of each powdered spice sample was weighed and extracted with 20 ml of acetonitrile–water (80:20 v/v) containing sodium chloride (NaCl) and magnesium sulfate ($MgSO_4$) to enhance the efficiency of toxin extraction. The mixture was shaken vigorously for 30 minutes and centrifuged at 4000 rpm for 10 minutes to separate solids from the extract. The supernatant was purified using immunoaffinity columns (IAC) designed to bind multiple mycotoxins or a C18 solid-phase extraction (SPE) column to remove interfering compounds. The purified extracts were filtered through 0.22 µm PTFE membrane filters before analysis. The purified extract was injected into a C18 reversed-phase column, and separation was performed using a gradient of water with 0.1% formic acid (Solvent A) and acetonitrile with 0.1% formic acid (Solvent B). The mass spectrometer was operated in electrospray ionization (ESI) mode and monitored multiple reaction monitoring (MRM) transitions specific to each mycotoxin. Calibration standards for aflatoxin B_1, B_2, G_1, G_2, and ochratoxin A were used to quantify the toxins in each sample. The ELISA technique was also employed for rapid screening of multiple mycotoxins using commercial multi-mycotoxin ELISA kits, which contained enzyme conjugates, specific antibodies, TMB substrate, PBS washing buffer, and 1 N H_2SO_4 stop solution. The sample extracts were diluted as recommended by the kit, and absorbance values were measured at 450 nm to determine concentrations of each mycotoxin.

3.4 Structural and Chemical Characterization of Detected Mycotoxins in Turmeric, Cloves, and Nutmeg

Approximately 5 g of each powdered spice sample was weighed and extracted with 20 ml of acetonitrile–water (80:20 v/v) containing sodium chloride (NaCl) and magnesium sulfate ($MgSO_4$) to enhance toxin recovery. The mixture was shaken vigorously for 30 minutes and centrifuged at 4000 rpm for 10 minutes to remove solids. The supernatant was purified using immunoaffinity columns (IAC) specific for multiple mycotoxins or C18 solid-phase extraction (SPE) columns to remove matrix interferences. The purified extracts were filtered through 0.22 µm PTFE membrane filters prior to instrumental analysis.

The purified extracts were injected into a C18 reversed-phase column, with separation achieved using a gradient of water with 0.1% formic acid (Solvent A) and acetonitrile with 0.1% formic acid (Solvent B). The mass spectrometer was operated in electrospray ionization (ESI) mode, and multiple

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reaction monitoring (MRM) transitions were used to detect each mycotoxin based on their unique precursor-to-product ion fragmentation patterns. The molecular weights, retention times, and fragmentation ions were compared with authentic standards of aflatoxin B_1, B_2, G_1, G_2, and ochratoxin A to confirm structural identity. LC-MS/MS data provided information on molecular formula, mass-to-charge ratio (m/z), and characteristic functional groups such as lactone rings, furan moieties, and coumarin structures in the detected mycotoxins.

ELISA was used for the biochemical confirmation of mycotoxins. Multi-mycotoxin ELISA kits containing specific antibodies, enzyme conjugates, TMB substrate, PBS washing buffer, and stop solution (1 N H_2SO_4) were employed. The ELISA procedure relied on the specific binding of antibodies to structural epitopes of each mycotoxin, providing selective detection of aflatoxins and ochratoxin A in the spice samples. The absorbance readings at 450 nm correlated with the concentration of each mycotoxin, confirming the presence of chemically active groups recognized by the antibodies.

3.5 Assessment of the Impact of Storage Conditions on Mycotoxin Accumulation in Turmeric, Cloves, and Nutmeg

Approximately 50 g of each powdered spice sample was divided into multiple aliquots and stored under controlled environmental conditions. Storage temperature was set at three levels: 25°C (ambient), 35°C, and 45°C. Relative humidity was controlled at 50%, 70%, and 90% using saturated salt solutions in desiccators to maintain stable moisture levels. Each sample set was stored for 0, 1, 2, and 3 months to monitor the effect of duration on mycotoxin accumulation. All samples were sealed in sterile, airtight containers to minimize external contamination while allowing humidity equilibration. At the end of each storage interval, the spice samples were extracted with acetonitrile–water (80:20 v/v) containing NaCl and $MgSO_4$ and purified using immunoaffinity columns (IAC) or C18 solid-phase extraction (SPE) columns. The purified extracts were filtered through 0.22 µm PTFE membrane filters. Mycotoxin concentrations were determined using LC-MS/MS, with separation on a C18 reversed-phase column and detection in electrospray ionization (ESI) mode using multiple reaction monitoring (MRM). Calibration standards for aflatoxin B_1, B_2, G_1, G_2, and ochratoxin A were used to quantify toxin levels in each sample.

IV. RESULTS

Table 4.1: Characterization of Spices

Spice	Moisture Content (%)	Water Activity (aw)	pH
Turmeric	8.5	0.52	5.5
Cloves	10.0	0.58	6.2
Nutmeg	7.2	0.48	6.5

The moisture content, water activity (a_n), and pH of turmeric, cloves, and nutmeg in table 4.1 indicated that their relative susceptibility to microbial growth and mycotoxin contamination. Turmeric (8.5% moisture, a_n 0.52, pH 5.5) and nutmeg (7.2% moisture, a_n 0.48, pH 6.5) have relatively low water activity, suggesting limited availability of free water for fungal proliferation, which may reduce the risk of mycotoxin formation under proper storage. Cloves, with higher moisture content (10%) and water activity (0.58), combined with a moderately acidic to neutral pH (6.2), are slightly more prone to fungal growth and mycotoxin accumulation, particularly under warm and humid conditions.

Table 4.2: Detection and Quantification of Mycotoxins

Spice Sample	Mycotoxin	ELISA (µg/kg)	LC-MS/MS (µg/kg)	Remarks
Turmeric	Aflatoxin B_1	5.2	5.0	Within permissible limit
Turmeric	Aflatoxin B_2	1.3	1.2	Trace level detected
Turmeric	Aflatoxin G_1	0.8	0.7	Low contamination
Turmeric	Aflatoxin G_2	0.5	0.4	Low contamination

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Turmeric	Ochratoxin A	2.1	2.0	Within safe limit
Cloves	Aflatoxin B_1	6.5	6.3	Slightly higher contamination
Cloves	Aflatoxin B_2	1.7	1.6	Trace level
Cloves	Aflatoxin G_1	0.9	0.8	Low
Cloves	Aflatoxin G_2	0.6	0.5	Low
Cloves	Ochratoxin A	3.0	2.8	Within safe limit
Nutmeg	Aflatoxin B_1	4.0	3.9	Safe
Nutmeg	Aflatoxin B_2	1.0	0.9	Trace
Nutmeg	Aflatoxin G_1	0.7	0.6	Low
Nutmeg	Aflatoxin G_2	0.4	0.3	Low
Nutmeg	Ochratoxin A	1.5	1.4	Safe

The analysis of mycotoxins in turmeric, cloves, and nutmeg using ELISA and LC-MS/MS in table 4.2 revealed the presence of multiple aflatoxins (B_1, B_2, G_1, G_2) and ochratoxin A in all spice samples, albeit at varying concentrations. In turmeric, aflatoxin B_1 and ochratoxin A were detected at 5.0 µg/kg and 2.0 µg/kg, respectively, which are within permissible limits, while aflatoxins B_2, G_1, and G_2 were present at trace or low levels, indicating minimal contamination. Cloves showed slightly higher contamination, with aflatoxin B_1 reaching 6.3 µg/kg and ochratoxin A 2.8 µg/kg, though still within safe limits, while the other aflatoxins were detected at low concentrations.

Source of Variation	Sum of Squares (SS)	df	Mean Square (MS)	F
Between Groups	2.61	2	1.30	F_{cal} =0.34
Within Groups	45.83	12	3.82	
Total	48.44	14		
				$F_{critical}$ =3.89

Critical F value at $\alpha = 0.05$

Since, F_calculated (0.34)<F_critical (3.89) the null hypothesis is accepted. This indicated that there is no statistically significant difference in the mean mycotoxin levels among turmeric, cloves, and nutmeg ($p > 0.05$). Although cloves recorded slightly higher contamination levels compared with turmeric and nutmeg, the variation observed among the spices was not large enough to be statistically significant. This implies that the contamination pattern is relatively similar across the three spice types, suggesting that the presence of mycotoxins may be influenced more by common factors such as storage conditions, moisture levels, and fungal exposure during processing and marketing, rather than by the spice type alone.

Table 4.3: Co-occurrence patterns of multiple mycotoxins

Spice Sample	Mycotoxins Detected	ELISA Concentration (µg/kg)	LC-MS/MS Concentration (µg/kg)	Co-occurrence Pattern	Remarks
Turmeric	AFB_1, AFB_2, OTA	5.1, 1.2, 2.0	5.0, 1.1, 1.9	AFB_1 + AFB_2 + OTA	Within permissible limits
Turmeric	AFG_1, AFG_2	0.8, 0.5	0.7, 0.4	AFG_1 + AFG_2	Low contamination
Cloves	AFB_1, OTA	6.4, 2.9	6.3, 2.8	AFB_1 + OTA	Slightly higher contamination
Cloves	AFB_2, AFG_1, AFG_2	1.6, 0.9, 0.5	1.5, 0.8, 0.4	Multiple aflatoxins	Trace levels
Nutmeg	AFB_1, AFB_2, OTA	4.0, 1.0, 1.5	3.9, 0.9, 1.4	AFB_1 + AFB_2	Safe

Nut meg	AFG_1, AFG_2	0.7, 0.3	0.6, 0.3	+ OTA AFG_1 + AFG_2	Low contam ination

The co-occurrence analysis of multiple mycotoxins in turmeric, cloves, and nutmeg in table 4.3 indicated that these spices are frequently contaminated with more than one toxin simultaneously. Turmeric and nutmeg showed combinations of aflatoxins B_1, B_2, and ochratoxin A, while AFG_1 and G_2 occurred at lower concentrations, suggesting a generally low risk of toxic synergy. Cloves exhibited slightly higher contamination levels, particularly with aflatoxin B_1 and ochratoxin A, and multiple aflatoxins were detected at trace levels. The presence of co-occurring mycotoxins, even at low concentrations, underscores the potential for additive or synergistic toxic effects, emphasizing the importance of monitoring and controlling storage and handling conditions to minimize health risks associated with spice consumption.

Table 4.4: Structural and Chemical Characterization of Detected Mycotoxins

Spice Sample	Mycotoxin	Molecular Weight (g/mol)	Retention Time (min)	Key Fragmentation Ions (m/z)	Characteristic Functional Groups	ELISA Confirmation (Absorbance at 450 nm)	Remarks
Turmeric	Aflatoxin B_1	312.27	12.5	313 → 241, 313 → 285	Lactone ring, furan moiety	1.25	Confirmed
Turmeric	Aflatoxin B_2	314.27	13.2	315 → 259, 315 → 287	Lactone ring, dihydrofuran	0.65	Confirmed
Turmeric	Aflatoxin G_1	328.27	14.0	329 → 243, 329 → 311	Lactone ring, furan, coumarin	0.85	Confirmed
Turmeric	Aflatoxin G_2	330.27	14.8	331 → 245, 331 → 313	Lactone ring, dihydrofuran, coumarin	0.55	Confirmed
Turmeric	Ochratoxin A	403.81	15.5	404 → 239, 404 → 358	Iso-coumarin, amide	1.10	Confirmed
Cloves	Aflatoxin B_1	312.27	12.6	313 → 241, 313 → 285	Lactone ring, furan moiety	1.30	Confirmed
Cloves	Aflatoxin B_2	314.27	13.3	315 → 259, 315 → 287	Lactone ring, dihydrofuran	0.70	Confirmed
Cloves	Aflatoxin G_1	328.27	14.1	329 → 243, 329 → 311	Lactone ring, furan, coumarin	0.90	Confirmed
Cloves	Aflatoxin G_2	330.27	14.9	331 → 245, 331 → 313	Lactone ring, dihydrofuran, coumarin	0.60	Confirmed
Cloves	Ochratoxin A	403.81	15.6	404 → 239, 404 → 358	Iso-coumarin, amide	1.15	Confirmed
Nutmeg	Aflatoxin B_1	312.27	12.4	313 → 241, 313 → 285	Lactone ring, furan moiety	1.20	Confirmed
Nutmeg	Aflatoxin B_2	314.27	13.1	315 → 259, 315 → 287	Lactone ring, dihydrofuran	0.60	Confirmed
Nutmeg	Aflatoxin	328.27	14.0	329 → 243,	Lactone ring,	0.80	Confirmed

	G_1			329 → 311	furan, coumarin		
Nutmeg	Aflatoxin G_2	330.27	14.7	331 → 245, 331 → 313	Lactone ring, dihydrofuran, coumarin	0.50	Confirmed
Nutmeg	Ochratoxin A	403.81	15.4	404 → 239, 404 → 358	Iso-coumarin, amide	1.05	Confirmed

The structural and chemical characterization of mycotoxins in turmeric, cloves, and nutmeg in table 4.4 revealed that all detected toxins were successfully identified and confirmed using both LC-MS/MS and ELISA. Aflatoxins B_1, B_2, G_1, G_2, and ochratoxin A were present in all three spices, with molecular weights ranging from 312.27 g/mol (AFB_1) to 403.81 g/mol (ochratoxin A). Retention times during LC separation varied slightly among spices, reflecting minor matrix effects, while key fragmentation ions confirmed the identity of each toxin. Characteristic functional groups, including lactone rings, furan moieties, coumarin structures, and iso-coumarin amides, were consistent with authentic standards, ensuring accurate structural confirmation. ELISA absorbance readings at 450 nm further validated the presence of chemically active groups recognized by specific antibodies, corroborating the LC-MS/MS results. These findings confirm that the spices contain structurally intact and biologically active mycotoxins, highlighting the need for monitoring to prevent potential health risks associated with consumption.

Table 4.5: Impact of Storage Conditions on Mycotoxin Accumulation

Spice Sample	Mycotoxin	Storage Temp (°C)	Relative Humidity (%)	Storage Duration (Months)	LC-MS/MS Concentration (µg/kg)	Remarks
Turmeric	Aflatoxin B_1	25	50	0	4.8	Initial level
Turmeric	Aflatoxin B_1	25	50	1	5.2	Slight increase
Turmeric	Aflatoxin B_1	25	50	2	5.8	Moderate increase
Turmeric	Aflatoxin B_1	25	50	3	6.5	Noticeable accumulation
Turmeric	Aflatoxin B_1	35	70	3	8.2	High accumulation due to heat & humidity
Turmeric	Ochratoxin A	45	90	3	10.5	Significant accumulation
Cloves	Aflatoxin B_2	25	50	0	1.1	Initial level
Cloves	Aflatoxin B_2	35	70	2	2.5	Moderate increase
Cloves	Ochratoxin A	45	90	3	6.8	High accumulation
Nutmeg	Aflatoxin G_1	25	50	0	0.8	Initial level
Nutmeg	Aflatoxin G_1	35	70	1	1.2	Slight increase

Nutmeg	Aflatoxin G_1	45	90	3	3.0	Noticeable accumulation
Nutmeg	Aflatoxin G_2	45	90	3	2.5	High accumulation
Nutmeg	Ochratoxin A	35	70	2	4.0	Moderate accumulation

The assessment of the impact of storage conditions on mycotoxin accumulation in turmeric, cloves, and nutmeg in table 4.5 demonstrated that temperature, relative humidity, and storage duration significantly influence toxin levels. In turmeric, aflatoxin B_1 gradually increased from 4.8 µg/kg at initial storage to 6.5 µg/kg after 3 months at 25°C and 50% humidity, with higher temperatures and humidity (35°C, 70% RH) causing more pronounced accumulation (8.2 µg/kg). Ochratoxin A in turmeric reached 10.5 µg/kg under the harshest conditions (45°C, 90% RH, 3 months), indicating that elevated heat and moisture accelerate toxin formation. Similar trends were observed in cloves and nutmeg, where aflatoxins B_2, G_1, G_2, and ochratoxin A increased with higher temperature, humidity, and prolonged storage.

V. DISCUSSION

The results in Table 4.1 demonstrate that turmeric, cloves, and nutmeg possess physicochemical properties that influence their susceptibility to microbial growth and mycotoxin contamination. Turmeric exhibited a moisture content of 8.5%, water activity (a_n) of 0.52, and pH 5.5, while nutmeg recorded lower moisture (7.2%), water activity (0.48), and slightly higher pH (6.5). These values suggest that both turmeric and nutmeg have relatively low water availability, limiting fungal proliferation and reducing the risk of mycotoxin formation when stored under appropriate conditions. In contrast, cloves showed higher moisture content (10%) and water activity (0.58) with a moderately acidic to near-neutral pH of 6.2, indicating slightly greater susceptibility to fungal growth and potential mycotoxin accumulation, particularly under warm and humid storage environments. The observed variations in water activity and pH are consistent with studies by Magan and Aldred (2021), who reported that spices with lower water activity (<0.6) are less conducive to fungal colonization and mycotoxin production. Similarly, Eskola et al. (2020) highlighted that elevated moisture and water activity facilitate fungal metabolism and enhance the likelihood of aflatoxin and ochratoxin formation in stored plant materials. The findings of Clasen et al. (2019) further support that the physicochemical environment of spices, including pH and water activity, critically determines the composition and density of fungal communities, which directly influences mycotoxin contamination levels. Furthermore, the results align with the work of Jayasinghe et al. (2022), who observed that spices with water activity values below 0.55 generally exhibit limited fungal growth, whereas those exceeding 0.55 are more prone to contamination, particularly by Aspergillus and Penicillium species. The relatively higher water activity of cloves may therefore facilitate the establishment of these fungi, increasing contamination risk. In addition, the pH values recorded in this study are consistent with those reported by Bhat et al. (2021), emphasizing that slightly acidic to neutral conditions favor certain toxigenic fungi, further explaining the relative vulnerability of cloves compared to turmeric and nutmeg.

The results in Table 4.2 indicate that turmeric, cloves, and nutmeg are contaminated with multiple aflatoxins (B_1, B_2, G_1, G_2) and ochratoxin A, although concentrations varied across spice types. In turmeric, aflatoxin B_1 and ochratoxin A were detected at 5.0 µg/kg and 2.0 µg/kg, respectively, both within permissible international limits, while aflatoxins B_2, G_1, and G_2 were present at trace or low levels, suggesting minimal contamination. Cloves showed slightly higher contamination, with aflatoxin B_1 reaching 6.3 µg/kg and ochratoxin A 2.8 µg/kg, still within safe limits, while the remaining aflatoxins were detected at low concentrations. Nutmeg exhibited the lowest overall contamination, with aflatoxin B_1 and ochratoxin A detected at 3.9 µg/kg and 1.4 µg/kg, respectively, reflecting relatively safe

levels for human consumption. These findings are consistent with the work of Demirhan and Demirhan (2023), who reported that aflatoxin B_1, B_2, G_1, and G_2 occur in turmeric samples, occasionally approaching or exceeding safety limits, emphasizing the need for monitoring. Juma et al. (2024) similarly observed detectable levels of aflatoxin B_1 in turmeric, although most samples were within permissible limits. Haq et al. (2024) reported that cloves could harbor aflatoxin B_1 and ochratoxin A, reflecting contamination during storage. Adeyeye and Oyedeji (2023) demonstrated that nutmeg samples contained measurable levels of aflatoxin B_1 and ochratoxin A, highlighting the impact of storage conditions on contamination levels. The detection of multiple mycotoxins in single samples aligns with Manda et al. (2022), who noted that co-occurrence of aflatoxin B_1 and ochratoxin A is common in spices, and underscores the importance of routine surveillance. The relationship between fungal contamination and toxin levels is further supported by Akinola et al. (2022), who showed that higher fungal counts in turmeric and nutmeg correspond to increased aflatoxin concentrations. Analytical methods used for quantification were reliable, with ELISA providing rapid screening and LC-MS/MS offering precise measurement of toxin levels, as confirmed by Chen et al. (2023) and Stroka et al. (2019).

The co-occurrence analysis in Table 4.3 revealed that turmeric, cloves, and nutmeg are frequently contaminated with multiple mycotoxins simultaneously, though at varying concentrations. Turmeric and nutmeg predominantly contained combinations of aflatoxins B_1, B_2, and ochratoxin A, while AFG_1 and AFG_2 were present at lower concentrations, indicating minimal risk of additive or synergistic toxicity. Cloves, however, exhibited slightly higher contamination levels, particularly with aflatoxin B_1 and ochratoxin A, and trace levels of multiple aflatoxins were also detected, suggesting a higher, though still manageable, potential for cumulative toxic effects. The findings of co-occurrence are consistent with Demirhan and Demirhan (2023), who reported simultaneous presence of aflatoxin B_1, B_2, G_1, and G_2 in turmeric samples, supporting the notion that multiple mycotoxins can exist in a single spice matrix. Juma et al. (2024) similarly confirmed co-occurrence of aflatoxin B_1 with other aflatoxins in turmeric, although concentrations were generally within permissible limits, aligning with the low-risk profile observed in this study. Haq et al. (2024) reported combined contamination of aflatoxin B_1 and ochratoxin A in cloves, corroborating the slightly higher contamination pattern observed here. The potential health implications of co-occurring mycotoxins are also supported by Adeyeye and Oyedeji (2023), who emphasized that nutmeg samples containing aflatoxin B_1, B_2, and ochratoxin A require careful monitoring despite low individual concentrations, as co-exposure could increase toxicological risks. This aligns with findings by Akinola et al. (2022), who demonstrated that higher fungal loads often correspond to the simultaneous presence of multiple toxins, particularly in spices with elevated moisture and water activity. Similarly, Silva et al. (2023) confirmed that even when individual mycotoxins were within safe limits, co-occurrence in turmeric and nutmeg could pose cumulative risks, supporting the necessity of comprehensive surveillance and control measures.

The structural and chemical characterization presented in Table 4.4 demonstrates that aflatoxins B_1, B_2, G_1, G_2, and ochratoxin A were successfully identified and confirmed in turmeric, cloves, and nutmeg using LC-MS/MS and ELISA. The molecular weights of the toxins ranged from 312.27 g/mol for aflatoxin B_1 to 403.81 g/mol for ochratoxin A, with retention times during liquid chromatography showing minor variations across spice matrices, likely due to subtle matrix effects. Key fragmentation ions observed in each sample (e.g., 313 → 241 for AFB_1 and 404 → 239 for OTA) matched those reported in authentic standards, confirming the identity of each mycotoxin. The detected functional groups, including lactone rings, furan moieties, coumarin structures, dihydrofuran, and iso-coumarin amides, further validated the chemical structures, supporting the reliability of the analytical methods used. ELISA absorbance values at 450 nm provided additional confirmation of biologically active groups recognized by specific antibodies, corroborating the LC-MS/MS results and indicating the presence of structurally intact, bioactive toxins. These findings align with Stroka et al. (2019), who reported that LC-MS/MS and ELISA provide complementary

validation for mycotoxin detection, ensuring high sensitivity and reproducibility across laboratories. Similarly, Chen et al. (2023) emphasized that structural characterization, including fragmentation ions and functional group identification, is essential for accurate confirmation of mycotoxins in complex food matrices such as spices. The co-occurrence of multiple mycotoxins in turmeric, cloves, and nutmeg, as confirmed structurally, also supports observations by Manda et al. (2022) and Demirhan and Demirhan (2023), highlighting the potential risk of simultaneous exposure to multiple toxins.

The results presented in Table 4.5 demonstrate that storage conditions including temperature, relative humidity, and duration, exert a profound influence on the accumulation of mycotoxins in turmeric, cloves, and nutmeg. In turmeric, aflatoxin B_1 increased gradually from an initial concentration of 4.8 µg/kg to 6.5 µg/kg after three months at moderate storage conditions (25°C, 50% RH), while exposure to higher temperature and humidity (35°C, 70% RH) accelerated accumulation to 8.2 µg/kg. Similarly, ochratoxin A in turmeric reached 10.5 µg/kg after three months at 45°C and 90% relative humidity, highlighting the susceptibility of this spice to toxin accumulation under unfavorable storage conditions. Cloves exhibited comparable patterns, with aflatoxin B_2 increasing from 1.1 µg/kg to 2.5 µg/kg under elevated temperature and humidity, and ochratoxin A reaching 6.8 µg/kg after prolonged exposure to high stress conditions. Nutmeg also showed increased levels of aflatoxins G_1, G_2, and ochratoxin A with prolonged storage under elevated temperature and relative humidity, reflecting a cumulative effect of environmental factors on mycotoxin formation. These findings are consistent with the work of Demirel et al. (2021) and Hassan et al. (2023), who reported that elevated temperature and relative humidity significantly enhance fungal growth and mycotoxin production in stored spices. The progressive increase in toxin concentration with storage duration aligns with observations by Demirhan and Demirhan (2023) and Juma et al. (2024), emphasizing that prolonged storage without adequate environmental control elevates the risk of contamination. Similarly, Adeyeye and Oyedeji (2023) highlighted that improper storage conditions, especially high humidity and temperature, directly contribute to ochratoxin A and aflatoxin accumulation in nutmeg and other spices. The influence of storage conditions on toxin accumulation is further supported by Akinola et al. (2022), who demonstrated that higher fungal counts resulting from warm and humid environments correlate strongly with increased mycotoxin levels. Chen et al. (2023) and Stroka et al. (2019) also emphasized that monitoring both physical storage parameters and toxin concentrations using sensitive analytical techniques, such as LC-MS/MS and ELISA, is critical for ensuring food safety. The observed trends in this study clearly indicate that even spices initially within safe limits may exceed permissible thresholds if stored under suboptimal conditions, underlining the importance of maintaining controlled storage environments to mitigate mycotoxin risks.

VI. CONCLUSION

The findings of this study demonstrate that turmeric, cloves, and nutmeg are susceptible to fungal contamination and mycotoxin accumulation, influenced by their physicochemical properties and storage conditions. Physicochemical characterization (Table 4.1) indicated that spices with lower water activity and moisture content, such as turmeric and nutmeg, have reduced susceptibility to fungal proliferation and mycotoxin formation, while cloves, with slightly higher moisture and water activity, are more prone to contamination under warm and humid conditions. Mycotoxin analysis (Tables 4.2 and 4.3) revealed the presence of multiple aflatoxins (B_1, B_2, G_1, G_2) and ochratoxin A in all samples, with turmeric and nutmeg generally showing concentrations within permissible limits, whereas cloves exhibited slightly higher contamination levels. Co-occurrence patterns highlighted that multiple mycotoxins can be present simultaneously, raising the potential for additive or synergistic toxic effects even at low concentrations. Structural and chemical characterization (Table 4.4) confirmed that the detected mycotoxins were chemically intact and biologically active, with characteristic functional groups (lactone rings, furan, coumarin, and iso-coumarin amides) verified by LC-MS/MS and ELISA. The impact of storage conditions (Table 4.5) clearly demonstrated that elevated temperature, high relative humidity, and prolonged storage significantly

increased mycotoxin levels in all spices. Turmeric and nutmeg, initially within safe limits, showed noticeable accumulation of aflatoxins and ochratoxin A under high heat and moisture, while cloves exhibited the highest susceptibility, confirming that improper storage accelerates mycotoxin formation.

VII. RECOMMENDATIONS

Based on the findings, the following were recommended;

i. Spices should be stored in environments with controlled temperature and relative humidity to minimize fungal growth and mycotoxin accumulation.
ii. Routine surveillance of mycotoxin levels using LC-MS/MS and ELISA should be conducted, particularly for spices stored for extended periods or traded commercially, to ensure compliance with permissible limits.
iii. Reducing moisture content and maintaining low water activity in spices during storage and transportation can help prevent fungal proliferation and subsequent mycotoxin formation.
iv. Spices with higher susceptibility, such as cloves, should be stored separately under optimal conditions to prevent cross-contamination and rapid toxin accumulation.

Since multiple mycotoxins can co-occur even at low levels, assessing combined mycotoxin profiles should be part of routine quality control to address potential additive or synergistic toxic effects.

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