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EVALUATION OF *ASPERGILLUS NIGER* CONTAMINATION AND OCCURRENCE OF CITRININ IN RED CHILLI (*CAPSICUM ANNUUM*) SAMPLES

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ABSTRACT

Numerous Ascomycete fungi produce toxic, low-molecular-weight secondary metabolites known as mycotoxins. Mycotoxin contamination poses a global challenge to food safety, and growing regulatory expectations regarding the presence of mycotoxins in various products have spurred increased research into detecting these toxins in food and animal feed. Mycotoxin contamination has been reported in many significant spices, including chillies. However, most research has focused on aflatoxins as primary contaminants, highlighting the need to investigate other lesser-studied mycotoxins, such as citrinin and patulin. Consequently, the current study aimed to screen for fungal contamination in locally available red chilli varieties and detect the presence of mycotoxins. Random samples of red chilli were collected to isolate and identify the fungi responsible for producing mycotoxins. High-performance liquid chromatography (HPLC) techniques and Fourier transform infrared (FTIR) spectroscopy were employed to analyze the extracted mycotoxins qualitatively. Morphological and molecular characterization through 18S rRNA sequencing of the isolated samples confirmed the presence of *Aspergillus niger* in red chilli. HPLC and FTIR analyses of the red chilli samples confirmed the occurrence of citrinin. Very few studies have reported the production of Citrinin by *A. niger* in red chilli. Further research is necessary to conduct quantitative analyses and assess the effects of citrinin on human health.

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1 Introduction

Mycotoxins are low molecular weight secondary metabolites produced by various fungal genera, including *Aspergillus*, *Fusarium*, and *Penicillium* (Doughari 2015). These toxins develop when fungi grow on different substrates under suitable conditions (Richard 2007; Tola and Kebede 2016). Mycotoxins can contaminate various food and feed samples (Bennett and Klich 2003; Pickova et al. 2021; Dey et al. 2022). Research has identified the presence of over a hundred mycotoxins in various dietary samples, which affect cereals, vegetables, fruits (Pereira et al. 2014; Zhao et al. 2018; Li et al. 2020; Wokorach et al. 2021), herbs (Altyn and Twauzek 2020; Chen et al. 2020; Caldeirao et al. 2021), spices (Boonzaaijer et al. 2008), and beverages like wine and milk (Myresiotis et al. 2015; Omar 2016; Carballo et al. 2021; Rocha et al. 2023).

Feed contamination poses additional food safety risks, as mycotoxins can carry over into animal-derived products, potentially affecting human health (Marin et al. 2013; Omotayo et al. 2019). These toxins have been linked to various health concerns, including hepatotoxicity (Ruan et al. 2022), nephrotoxicity (Weidemann et al. 2016), genotoxicity (Theumer et al. 2018), neurotoxicity (Wang et al. 2024), and immunosuppression (Benkerroum 2020). The most commonly found mycotoxins include Fumonisins, Zearalenone (ZEA), Aflatoxins (AF), Ochratoxins (OT), Citrinin, Patulin, and Trichothecenes (Hove et al. 2016). The World Health Organization (WHO) has established the highest regulatory limits for mycotoxins at 10-30 µg/kg, highlighting the severe health risks they pose to humans and animals (Lee et al. 2017).

Various analytical techniques detect and measure mycotoxins in food samples, including rapid strip screening assays, immunoassay-based methodologies, and chromatographic techniques (Agriopoulou et al. 2020). Techniques such as Thin Layer Chromatography (TLC), High-Performance Liquid Chromatography (HPLC), Gas Chromatography-Tandem Mass Spectrometry (GC-MS), and Enzyme-Linked Immunosorbent Assay (ELISA) are also utilized for quick mycotoxin analysis due to their profound implications (Lee et al. 2013; Younis et al. 2020; Liew and Sabran 2022).

Control measures for mycotoxins primarily focus on effective agricultural practices and management approaches, including modifying antifungal genes, using biocontrol agents, and breeding plants for tolerance (Chatterjee et al. 2023). Additionally, government programs aim to remove contaminated commodities from the food chain as part of regulatory efforts (Chatterjee et al. 2023). With the growing demand for healthy meals and legislative expectations, defining mycotoxin contamination limits has become imperative (Chatterjee et al. 2023).

Red chillies (*Capsicum annuum* Linn.) are one of the most popular spices consumed globally and are the second-largest production spice (Samyal and Sumbali 2020). They are mainly produced in tropical and subtropical regions, with China, India, and Thailand accounting for nearly half of the world's production (FAOSTAT 2017; Eskola et al. 2020). In India alone, they are cultivated over 8,000 hectares, resulting in an annual production of 1,872,000 Metric Tons (MT) (DACFW 2018). However, red chillies are significant sources of microbial contamination, particularly high aflatoxin levels (Romagnoli et al. 2007).

The crop is susceptible to various fungal pathogens and can be attacked by mycotoxin-producing fungi during cropping, harvesting, and post-harvesting stages (Golge et al. 2013). Commercially sold red chillies often contain mycotoxin concentrations that exceed the maximum permissible limits. Citrinin (CIT) is the most frequently occurring mycotoxin produced by the genera *Penicillium*, *Aspergillus*, and *Monascus* (AjithKumar et al. 2023). CIT is a polyketide typically found in stored grains, vegetables, fruits, juices, herbs, spices, and spoiled dairy products (Doughari 2015). A literature review indicated that studies on citrinin contamination in red chillies are limited; therefore, the present study was conducted to screen for fungal contaminants in red chilli samples. Further qualitative analysis and identification of purified mycotoxin were performed using HPLC and Fourier Transform Infrared (FTIR) techniques.

2 Materials and Methods

2.1 Collection of red chilli samples

For this investigation, samples of mycotoxin-contaminated red chilli (*Capsicum annuum*) were identified and procured from local markets in Bangalore, Karnataka, India. Thirty-day-old red chilli samples were used in this study. All samples were collected and transported directly to the microbiological laboratory in an airtight container under aseptic conditions to prevent fungal contamination from external sources. The samples were surface sterilized using a 2.5% sodium hypochlorite solution for three minutes, rinsed multiple times in sterile double-distilled water, and dried (Rajarajan et al. 2013).

2.2 Isolation of mycoflora from the collected contaminated samples

Crushed red chilli samples (1 g) were suspended in 9 mL sterile distilled water. For each sample, an aliquot (0.1 mL) of the suspension was serially diluted in sterile water and then spread onto Potato Dextrose Agar (PDA) plates containing chloramphenicol. The plates were incubated at 25°C for 7 days. Rose Bengal agar medium supplemented with chloramphenicol was also used to isolate fungal colonies with distinctive physical

characteristics. After the incubation period, mold colonies on each plate were counted, and the colony-forming units (CFU) per gram were determined (Kutama et al. 2022).

2.3 Identification of fungal strains isolated from red chilli samples

Different morphologies of fungal colonies were selected based on their source, and pure cultures of each isolate were maintained on both PDA plates and slants. The colony characteristics, including colour and morphology, were recorded for the isolated strains. The phenotypic morphologies of the fungal colonies were examined using Lactophenol Cotton Blue (LPCB) staining. This method helped determine the size of the conidia and the characteristics of vesicles, conidiophores, and phialides, which are essential for identifying the genus and order of the parasitic organisms. Species-level identification was conducted through 18S rRNA sequencing at Barcode Biosciences in Bengaluru (Setlem and Ramlal 2022; Chen et al. 2020).

2.4 Screening of mycotoxin production by isolated fungal strains

2.4.1 Growth on different media

The fungal isolates were cultured on various media, including Coconut Cream Agar (CCA), Malt Extract Agar (MEA), Yeast Extract Sucrose Agar (YES), and Czapek Dox Agar (CZA). The resulting cultures were tested for fluorescence under UV light. The different colours emitted from the cultures indicate the presence or absence of various types of mycotoxins (Zhang et al. 2016).

2.4.2 Ammonia Vapour Test

The selected fungal isolates were cultured on YES medium and incubated at 28°C without light. After three days, the plates were exposed to ammonium hydroxide in a closed chamber (Sadhasivam et al. 2017). The change in colour of the culture medium indicated whether the isolates were toxic (Darab et al. 2010). After ten minutes, if the undersides of the agar plates turned pink-red, this indicated the presence of mycotoxin-producing isolates. In contrast, the isolates were considered non-toxic if no colour changed (Moradi et al. 2017).

2.5 Extraction of a mycotoxin from contaminated red chilli samples

Fifty grams of contaminated red chilli samples were taken and blended in a methanol and water solvent system in a 55:45 volume/volume ratio. The mixture was supplemented with 2 grams of sodium chloride and 50 milliliters of hexane (Agriopoulou et al. 2020). The samples were blended at high speed for 5 to 10 minutes and filtered through the Whatman No. 1 filter paper. After filtration, an equal volume of chloroform was added to the filtrate, and the mixture was vigorously agitated for 2 minutes before allowing it to rest for separation. The lower phase was collected, evaporated almost to dryness at 65 ˚C, and then redissolved in 10 milliliters of a methanol-water solvent system in an 80:20 volume/volume ratio (Setlam and Ramlal 2022).

2.6 Qualitative and quantitative analysis of mycotoxin extract

2.6.1 UV Spectrophotometric Analysis

UV analysis of the extracted mycotoxin sample, suspended in 1 ml of methanol, was conducted using a Vis spectrophotometer across wavelengths of 200-900 nm (Kumar and Suresh 2019). The spectrum was analyzed to identify the type of mycotoxin in the sample.

2.6.2 Thin Layer Chromatography (TLC)

To identify the presence of mycotoxins, 5 µL of the extracted toxin was applied to a 0.5 mm thick silica gel plate previously coated with glycolic acid. Standard mycotoxin solutions were also spotted on the same plate. The plate was then developed using a mobile phase consisting of 6 parts toluene, 3 parts ethyl acetate, and 1 part formic acid. After the development process, the plate was air-dried and examined under a UV lamp. The fluorescence intensities of the sample spots were recorded, and mycotoxins were identified based on the color and Retention Factor (Rf) of the spots compared to those of the standard spots. Additionally, further development of the spots was achieved through vapor exposure or immersion in aluminum chloride reagent, followed by viewing at a wavelength of 365 nm (Hongyin et al. 2021).

2.6.3 High-Performance Liquid Chromatography (HPLC)

The mycotoxin levels in the extract were assessed and measured using High-Performance Liquid Chromatography (HPLC) at the ALS Laboratory in Bengaluru, Karnataka, India. For the analysis, 500 µl of methanol was introduced into the HPLC system, equipped with a fluorescence detector (ALSIN Equipment). The column temperature was maintained at 25˚C. The excitation and emission wavelengths were set to 335 nm and 500 nm, respectively. A 50 µl sample of the mycotoxin was injected into the HPLC. To determine the optimal detection conditions for each toxin, the excitation and emission wavelengths were varied: Aflatoxin B1 was measured at 365 nm (excitation) and 440 nm (emission), Ochratoxin A at 335 nm and 465 nm, and citrinin at 331 nm and 500 nm. The mobile phase consisted of 0.25N phosphoric acid, acetonitrile, and propanol in a ratio of 550:350:10 (v/v/v). The instrument was operated under isocratic conditions with a 0.5 mL/min flow rate. The mycotoxins were identified by comparing the peak areas with standard mycotoxins' calibration curves, as Li et al. (2012) described.

2.6.4 Fourier Transform Infra Red (FTIR) analysis

FTIR analysis was conducted on a methanolic extract of contaminated red chilli samples at VIT, Vellore, Tamil Nadu, to determine the optimal detection conditions for each toxin. The analysis used an FT-IR spectrometer in Attenuated Total Reflectance (ATR) mode, covering a 400-4000 cm^{-1} range. The spectrum revealed various modes of vibration.

2.7 Statistical Analysis

The experiments were conducted in triplicate. Statistical analysis was performed using SPSS version 25.0. The results are expressed as the mean ± standard deviation (SD), with a statistical significance level of $P < 0.05$.

3 Results and Discussion

3.1 Isolation and identification of the mycoflora from red chilli samples

The analysis of red chilli samples for fungal contaminants identified a single type of fungal colony across all samples. The morphological characteristics, including colony color, shape, and size, were documented and are presented in Table 1. Microscopic examination revealed that the isolate belongs to the genus *Aspergillus* (Watanabe 2018).

The isolated fungal strain underwent 18S rRNA sequencing for further identification confirmation. The sequencing results were entered into the nucleotide BLAST program via the NCBI database to identify the isolates. Based on these sequences, a phylogenetic tree was constructed using MEGA software (Figure 1). Morphological and microscopic observations, along with the 18S rRNA sequencing, led to the identification of the isolated fungal strain as *Aspergillus niger*. The occurrence of *A. niger* in red chilli has been reported in only a few recent studies by Rajendran et al. (2021), Tsehaynesh et al. (2021), Enamullah et al. (2002), and Lasram et al. (2022). In contrast, many studies have cited the prevalence of other members of the *Aspergillus genus*, such as *A. flavus* and *A. parasiticus*, in red chilli (Hossain et al. 2018; Rajendran et al. 2021; Darsana and Chandrasehar 2021). Although mycotoxin contamination is a concern in countries that produce red chilli (Singh and Cotty 2017), it has not been extensively studied in previous research.

3.2 Screening tests for mycotoxin production by isolated fungal strains

When the isolated strains were cultured on various media, including CCA, MEA, YES, and CZA, and exposed to UV light, green fluorescence indicated the presence of mycotoxins (Table 2) (Agriopoulou et al. 2020). Similarly, Rajarajan et al. (2021) reported the production of fluorescent pigments in coconut milk agar (CMA) by aflatoxin-producing *A. flavus*. This fluorescence may be attributed to the influence of coconut on the production of the fluorescent pigment.

3.3 Ammonium Vapour Test

Isolates grown on Potato Dextrose Agar exhibited pink pigmentation when exposed to ammonium vapor. This color change indicates the presence of mycotoxins, as ammonium vapor interacts with these toxins to produce pink to red pigmentation (Shekhar et al. 2017; Rajarajan et al. 2021).

Table 1 Colony morphology and microscopic observation of mycoflora isolated from red chilli samples

Colony Characteristics	Microscopic Morphology	Identified genus	Reference
Colonies had a cottony appearance and were grey to black on the top with a whitish reverse side.	The conidial head is biseriate and radiate, with conidia arranged in chains or dispersed when detached.	*Aspergillus* spp.	Watanabe 2018

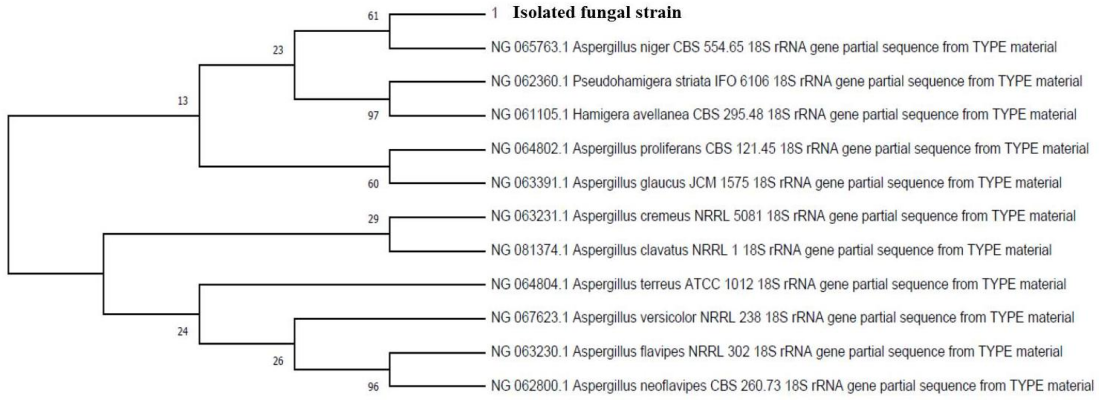

Figure 1 Phylogenetic tree of a fungal strain isolated from red chilli

Table 2 UV Fluorescence of Isolated Fungal Culture Cultivated in Different Media

Sample Source	Isolated Fungi	Media			
		CCA	MEA	CZA	YES
Red Chilli	*Aspergillus* spp.	+	+	-	+

CCA- Coconut Cream Agar, MEA- Malt Extract Agar, CZA-Czapek Dox Agar, YES – Yeast Extract Sucrose Agar

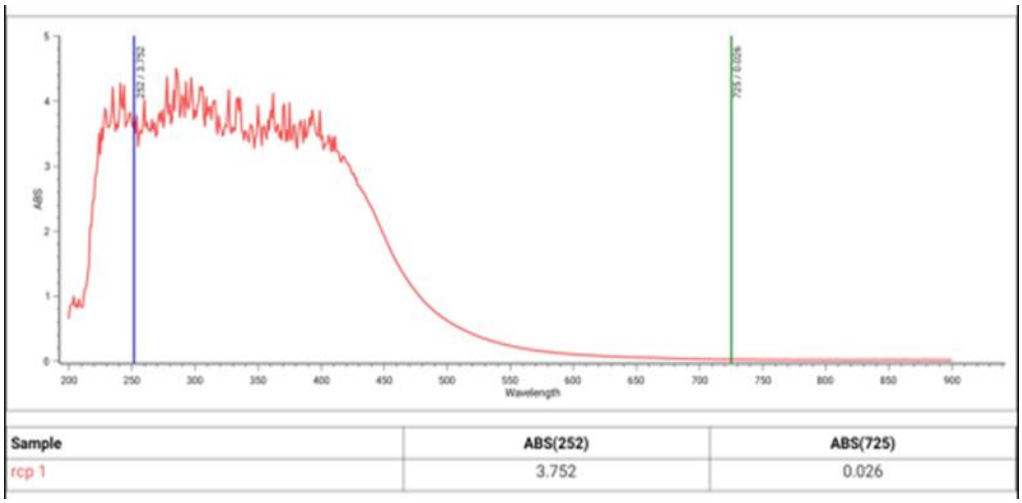

Figure 2 UV Spectrophotometric analysis of mycotoxin extracted from red chilli sample

3.4 Qualitative and quantitative analysis of mycotoxin extract

3.4.1 UV Spectrophotometric Analysis

The UV spectrum analysis of the mycotoxin extracts revealed a peak value of 252 nm, with an absorbance of 3.752 (Figure 2). Similar peaks at this wavelength were reported in mycotoxins from date palms by Kumar and Suresh (2019) and from animal feed by Rajarajan et al. (2021). Samyal and Sumbali (2021) and Akintola et al. (2024) have also documented the production of mycotoxins by various fungal species in red chilli.

3.4.2 Thin-layer chromatography (TLC) analysis

The thin-layer chromatography (TLC) plates observed under 365 nm light showed lemon yellow spots in the current investigation. The fluorescence of these spots was compared to standard mycotoxins, and the Rf value of the spot was determined to be 0.63. Based on this comparison, the mycotoxins produced by *Aspergillus niger* in this study were identified as Citrinin (Rasheva et al. 2003; Doughari 2015). Since the spots were faint and fragile, the TLC plate was sprayed with aluminum chloride, which caused the spots to change from yellow to blue. Additionally, incorporating glycolic acid into the silica gel enhanced the visibility of the spots with minimal diffusion, thus improving detectability. Mycotoxin detection using TLC is often semi-quantitative or quantitative, with a visual identification recovery limit of 0.01 ppm. Rasheva et al. (2003) reported that silica gel treated with organic acid could detect various types of mycotoxins, including citrinin, aflatoxin, and fumonisin.

3.4.3 HPLC Analysis

HPLC is commonly used to detect mycotoxins. In the current study, a solvent composed of 0.25N phosphoric acid, acetonitrile, and 2-propanol in a ratio of 55:35:10 successfully eluted mycotoxins as a sharp peak within 3 minutes. This retention time confirmed the presence of the mycotoxin citrinin in the tested sample (Figure 3). Recently, citrinin was also reported in date palms infected with *A. niger* (Schmidt-Heydt et al. 2012; Sadhasivam et al. 2021). Among all *Aspergillus* species, *A. niger* is primarily responsible for the production of citrinin. While *A. niger* is the leading producer, other *Aspergillus* species such as *A. awamori*, *A. wentii*, *A. fumigatus*, *A. niveus*, *A. ostianus*, and *A. parasiticus* are also known to produce Citrinin (Doughari 2015). Similarly, Samyal and Sumbali (2020 & 2021) reported that some fungal pathogens, including *A. terreus*, *Penicillium expansum*, and *P. fellutanum*, produce citrinin in red chilli. Citrinin has a conjugated planar structure with natural fluorescence, making it detectable by reverse-phase HPLC using phosphoric acid as a mobile phase in food commodities (Wang et al. 2014). Li et al. (2012) reported that citrinin can be detected at concentrations as low as 2–5 ng, with a higher retention time and peak area. Mycotoxins are undesirable toxins produced by pathogenic fungi in poorly stored or processed spices, making their detection crucial. Therefore, HPLC combined with UV-VIS or MS/MS

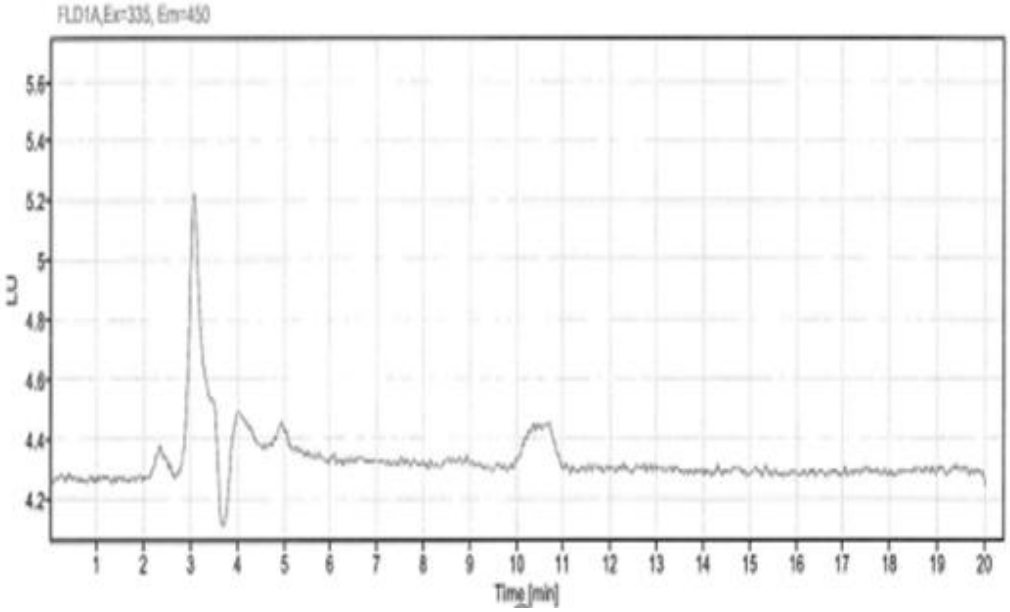

Figure 3 HPLC analysis for red chilli sample

Table 3 FTIR spectroscopic data of mycotoxin extracted from red chilli samples

S. N.	Obtained Peak (Wave number cm-1)	Frequency Range (Wave number cm-1)	Peak intensity	Absorption Band	Mode of vibration and Functional group	References
1.	3269	2500-4000	Weak	Broad	Hydrogen Bond	Kaya-Celiker et al. (2014); Dandashire and Almajir (2020)
2.	2924	2500-4000	Medium	Sharp and narrow	Aromatic –C-H and CH_2	
3	2852	2500-4000	Weak	Sharp and narrow	Aromatic –C-H and CH_2	
4.	1739	1700-1800	Medium	Sharp	C=O stretching of carbonyl groups (ketones, esters, or acids)	
5.	1627	1485-1690	Weak	Broad	C=O, C-N stretching of amide, N-H, C-O bending and C-C, C-N stretching of amide	
6.	1371	1330-1410	Weak	Broad	Symmetric C-H bending of methyl group	
7.	1234	600-1500	Weak	Sharp	CH_2 – Bending vibration	
8.	1155	1000-2000	Weak	Sharp	C-O, CH_2 stretching, bending	
9.	1018	1000-2000	Strong	Broad	C-O, CH_2 stretching, bending	

detectors has proven effective for determining mycotoxins in various species (Sahu et al. 2023).

3.4.4 FTIR Analysis

FTIR is a rapid, reliable, and sensitive technique for detecting mycotoxins in food samples. The FTIR results of the analyzed samples confirmed the presence of mycotoxins, as evidenced by the peaks and wave numbers specific to the functional groups of mycotoxins found in their FTIR spectra. These peaks were documented, and the modes of vibration and the functional groups were analyzed, as outlined in Table 3. The FTIR analysis of a contaminated sample of dried red chilli revealed the presence of mycotoxins, indicated by peaks in the FTIR spectrum (Figure 4). These peaks correspond to the characteristic functional groups associated with mycotoxins, including ranges from 2500-4000 cm^{-1}, 1700-1800 cm^{-1}, 1485-1690 cm^{-1}, 1330-1410 cm^{-1}, 600-1500 cm^{-1}, and 1000-2000 cm^{-1}. Specifically, these involve

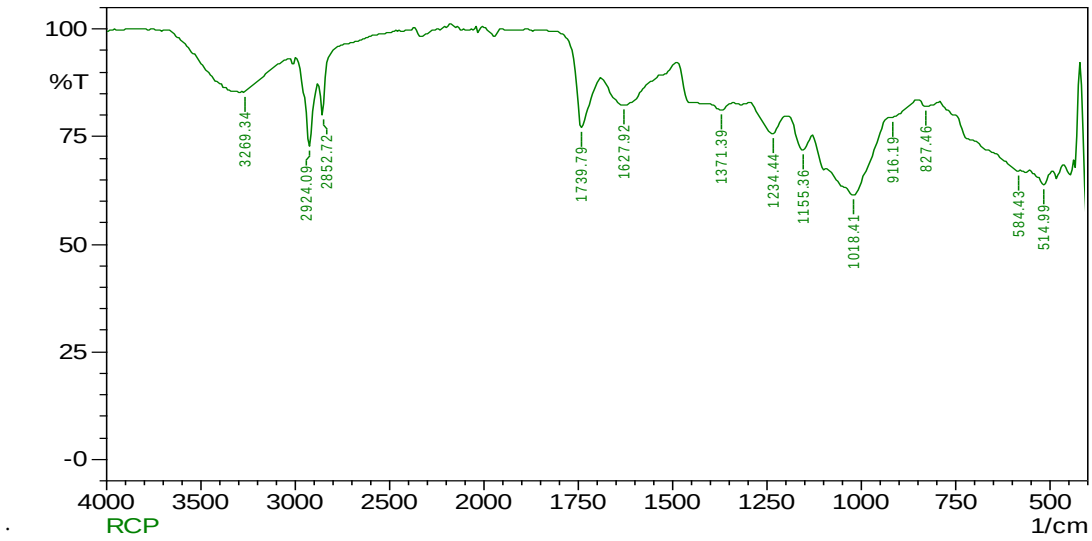

Figure 4 FTIR spectra of the mycotoxin extracted from red chilli samples

Aromatic –C-H and CH_2 stretching, C=O stretching of carbonyl groups (such as ketones, esters, or acids), C-N stretching of amides, N-H bending, C-O bending, symmetric C-H bending of methyl groups, and CH_2-bending vibrations. FTIR spectroscopy is a nondestructive method for measuring the total amount of infrared energy absorbed by the sample under investigation. This user-friendly technique can evaluate sample depths ranging from 10 µm to 100 mm with minimal interference from surface light scattering (Lomont et al. 2022). The primary advantage of FTIR is its ability to perform depth profiling for the nondestructive evaluation of food items. Additionally, mycotoxins' wavelength and absorption patterns were similar in studies conducted by Kaya-Celiker et al. (2014) and Dandashire and Almajir (2020).

Recent reports indicate natural contamination of citrinin (CIT) in regional spices, with the highest concentrations found in dried ginger, black pepper, and red chilli, reaching up to 47.2%. Dried ginger exhibited the most significant average concentration of CIT at 85.1 µg/kg (Yogendrarajah 2015; Mair et al. 2021). A dietary exposure study conducted in Belgium by Meerpoel and Vidal (2021) revealed that 50% of the herbs and spices tested were contaminated with CIT, with the highest concentration recorded at 4.5 µg/kg. In 2019, the maximum regulatory limit for CIT was reduced from 2000 µg/kg to 100 µg/kg following an amendment to Section 2.8.1 of the Annex to Regulation (EC) 1881/2006. This change was related to EU regulations on red yeast rice food supplements. Recent European monitoring studies have detected CIT and its associated biomarkers. However, among the 72 recognized methods for mycotoxin analysis in food published by the International Organization for Standardization (ISO), the European Committee for Standardization (CEN), and the Association of Official Agricultural Chemists (AOAC), none have specifically targeted CIT (Ali and Degen 2019).

Conclusion

Mycotoxins are significant contaminants found in food and feed, threatening human and animal health, food safety and agriculture. This issue remains a concern despite global efforts to reduce or eliminate their presence. Public health authorities and governmental bodies are implementing strict regulations for the most commonly found classes of mycotoxins in food and feed. Various analytical techniques have been developed to minimize mycotoxin levels; however, the problem persists. This study investigates fungal contamination-producing mycotoxins in red chilli. Molecular characterization confirmed the presence of *A. niger*, and analytical methods such as Thin Layer Chromatography (TLC), High-Performance Liquid Chromatography (HPLC), and Fourier-Transform Infrared Spectroscopy (FTIR) identified the production of citrinin by the mycoflora in red chilli. This research reports the presence of citrinin produced by *A. niger* in red chilli. The combined detection approach provides better insights into recognizing the potential risks of mycotoxin contamination. It also paves the way for developing ultra-sensitive detection techniques, ensuring high-quality, safe, and contaminant-free food. Additionally, this paper emphasizes the importance of precautionary measures to be taken during the production, handling, and transportation of spices to reduce mycotoxin accumulation.

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Competing interests

The authors declare no competing interests.

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