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Research article

Toxigenic mycofloral association and mycotoxins contamination in turmeric, coriander & red chilli and risk assessment of its effect

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Abstract

This study was aimed to determining the presence of aflatoxiginic, ochratoxigenic and citrinin producing fungi and their mycotoxins production in turmeric, coriander and red chilli from Bihar state (India). A. flavus were the most dominant species present in all types of spices. Red chilli has the highest incidence of A. flavus (32.3) followed by turmeric (28.3). 56 % of A. flavus from red chilli and 40% of A. ochraceus from turmeric were toxigenic and produced aflatoxins and ochratoxin A respectively. Qualitative and quantitative detection of mycotoxins were analyzed by LC-MS/MS. 85.4% of red chilli and 73.3% of coriander samples were contaminated with aflatoxins. The maximum amount of aflatoxins was detected in red chilli (219.6 ng/g), ochratoxin A was in turmeric (125.9 ng/g) and citrinin was in coriander samples (81.0 ng/g). The result of this study suggest that examined spices are susceptible substrate for growth of mycotoxigenic fungi and further mycotoxin production and the amount present were sufficiently high to induce carcinogenesis. This is the first report of natural occurrence of citrinin in spices from Bihar state, India.

Keywords - aflatoxins, ochratoxin A, citrinin, toxigenic fungi, LC-MS/MS.

Introduction

India is the largest spice producer country in the world and about 68 different spices are cultivated here. India has many different climatic zone whose temperature varies from 20°C to 28°C in which different spices are cultivated and unfortunately this temperature is the optimum temperature for the growth of fungi and mycotoxin productions. Further handling, transportation, poor processing and storage system enhances contamination and quality deterioration in the spices. Mycofloral contamination of spices generally occurs when they are not dried properly or stored in humid environment. Mycotoxins are the secondary metabolites of fungi produced on wide range of foods and feeds. The most common fungal contaminants of spices are belonging to *Aspergillus*, *Penicillium* and *Fusarium* genera. Some species of these genera have potential to produce different mycotoxins such as aflatoxins, ochratoxin and citrinin etc. Aflatoxins are naturally occurring secondary metabolites from the some species of *Aspergillus* genus and they are carcinogenic [1]. OTA are produced by the *A. ochraceus* and *P. verrucosum* and also by some other species. CTN is generally produced by *P. citrinum*, *A. ochraceus* and *A. terreus.* OTA is carcinogenic and causes hepatorenal carcinogenesis [2] and CTN is nephrotoxic and which also affects the immune system in animals [3].

Spices are widely used as ingredient in food preparation which provides distinctive color, flavor and aromas. Turmeric, coriander and red chilli are important spices widely used in cooking especially in north India. They are rich in carbohydrates and proteins whereas some other nutrients also present like vitamin A, vitamin K, vitamin C, magnesium, phosphorus and antioxidants compounds and have also the medicinal properties in the ayurveda therapy [4].

Some fragmentary reports are available regarding mycoflora and mycotoxins contamination in spices from India and different part of the world. However these reports are mainly confined to aflatoxins contamination in spices [5-7]. This is the first report regarding citrinin contamination in spices from Bihar state, India. Earlier Bilgrami and Jeswal [8] reported citrinin contamination in cereals from Bihar.

The present study was conducted to ascertain the predominant mycoflora associated with commonly used spices (turmeric, red chilli and coriander) of Bihar and natural occurrence of aflatoxins, ochratoxin A and citrinin. The presence of aflatoxins, ochratoxin A and citrinin in spices is a matter of concern because these mycotoxins can directly or indirectly affect the human health.

Material and Methods

Sampling

Samples were collected from the local markets of Bihar state (India) and coriander. Total 110 samples were collected (55 red chilli, 35 turmeric and 30 coriander) and put into the sterile cellophane bag then into the sterile brown envelop and stored at 4°C to arrest any mycotoxin formation before analysis.

Fungal isolation and identification

All the samples had randomly placed on the freshly prepared Potato dextrose agar (PDA) and standard Blotter paper and incubated at 28 ± 2°C for 7 days and examined daily. The counts were recorded after 5 to 7 days. After incubation all plates were examine visually and by binocular stereomicroscope. Fungal colonies of different morphological type were sub-cultured by hyphal tip method culture tube containing PDA media. Identification of fungi was carried out by morphological characteristics and followed the taxonomic schemes of Maren) [9] for genus *Aspergillus*; Pitt [10] for *Penicillium;* Leslic and Summerell [11] for *Fusarium* and Crous *et al* [12] for other genera.

Detection of potentiality of mycotoxins producing fungi

Aflatoxins producing potentiality of *Aspergillus* species were done by the method of Diener and Davis [13] using SMKY media. Schwenk *et al* [14] and Davis *et al* [15] methods were used for citrinin and ochratoxin A using YES media and extracted with chloroform. In case of citrinin the culture filtered was acidified with 1N HCL to bring down the pH subsequently then it was extracted with chloroform. The chloroform extract was evaporated to dryness and residue was dissolved in 1 ml of chloroform for further qualitative and quantitative estimation using TLC.

Natural occurrence of mycotoxins in spice samples by detected by LC-MS/MS

Natural occurrence of total aflatoxins, ochratoxin A and citrinin in spices samples were analyzed by LC-MS/MS using Aligents Poroshell 120 EC C18, 2.1x100mm column. 10 gm of sample were grinded and added 40 ml of extraction solution containing Acetonitrile : Water (40:10, v/v) and vortexed vigorously for 5 minute and then shake it gently for another 45 minutes. The solutions were filtered through 0.2µ Nylon syringe filter and the supernatant was collected. 2ml of filtrate was taken and dried under fine stream of N_2 gas. Add 1ml of reconstitute solution of Acetonitrile : Water (10:40, v/v) and vortexed. 0.5µl of sample was injected into LC-MS/MS (Agilent 6410) containing the mobile phase of 0.1% formic acid in 5mM ammonium acetate and methanol.

Results and Discussions

Association of toxigenic fungi

Prevalence of mycoflora were observed in turmeric, red chilli and coriander sample; total18 species of 7 different fungal genera were isolated (Table 1). *Aspergillus* and *Penicillium* was the most dominated genera and present in all 3 types of spices. *A. flavus* contamination was highest in red chilli (32.3) followed by turmeric (14.3) and coriander (8.0). Earlier, *A. flavus* and aflatoxins in spices from Oman were reported by Elshafie *et al* [16]. In our result, some of the fungi were only confined to specific spices. *A. alternata*, *A. tamari* and *C. globosum* species were only present in red chilli samples, *A. Parasiticus* and *F. oxysporum* were confined to turmeric and *A. fumigates*, *A. sydowi* and *R. nigricans* were only isolated from coriander samples. All these results indicate, it may be possible that some of the specific nutrients or essential oil present in these spices supports the growth of specific fungi or inhibit the growth of other fungi. It has been observed that all 3 spices (red chilli, turmeric and coriander) are susceptible substrate for growth of *A. flavus*, *P. citrinum*, *P. verrucosum* and *M. hiemalis.* (Fig. 1).

Table 1: Percent incidence of isolated fungi, pH and moisture content of spices.

Fungal species	Name of the spices		
	Red chilli	Turmeric	Coriander
Alternaria alternata	2.3	-	-
Aspergillus parasiticus	-	3.1	-
Aspergillus tamarii	5.3	-	-
Aspergillus niger	15.3	-	10.6
Aspergillus flavus	32.3	14.3	8.0
Aspergillus ochraceus	8.6	5.4	-
Aspergillus versicolor	11.42	-	-
Aspergillus fumigatus	-	-	2.0
Aspergillus sydowi	-	-	2.6
Penicillium citrinum	12.6	13.8	7.6
Penicillium verrucosum	8.6	6.5	6.3
Fusarium oxysporum	-	6.5	-
Fusarium moniliforme	6.3	4.1	-
Chaetomium globosum	1.40	-	-
Rhizopus nigricans	-	-	4.3
Rhizopus oryzae	4.3	5.9	-
Mucor hiemalis	7.0	4.8	2.6

Table 2: Detection of Mycotoxin producing potentiality of toxigenic fungi isolated from different spices.

S. No.	Sample	Fungi examined	Positive/N.I.A[a]	% toxicity	Mycotoxin detected	Potential range (µg/l)
1.	Red chilli	*Aspergillus flavus*	14/25	56.0	Aflatoxins	4.3 – 33.6
		Aspergillus ochraceus	5/12	41.6	Ochratoxin A	2.8 – 18.6
		Penicillium citrinum	5/16	31.2	Citrinin	15.6 – 28.3
		Penicillium verrucosum	3/10	30.0	Ochratoxin A	3.5 – 8.6
3.	Turmeric	*Aspergillus flavus*	4/20	20.0	Aflatoxins	4.4 – 11.3
		Aspergillus parasiticus	6/15	40.0	Aflatoxins	1.0 – 16.8
		Aspergillus ochraceus	6/15	40.0	Ochratoxin A	2.1 – 18.9
		Penicillium citrinum	0/15	0	-	-
		Penicillium verrucosum	2/12	16.6	Ochratoxin A	3.7 – 6.1
4.	Coriander	*Aspergillus flavus*	7/18	38.8	Aflatoxins	5.2 – 13.7
		Penicillium citrinum	4/15	26.6	Citrinin	18.7 – 20.9
		Penicillium verrucosum	3/15	20	Ochratoxin A	6.8 – 16.1

[a]Number of Isolate analyzed.

Mycotoxin producing potentiality of isolated fungi

Aflatoxins, ochratoxin A and citrinin producing potentiality and toxicity of *A. flavus*, *A. parasiticus*, *A. ochraceus*, *A. terreus*, *P. citrinum* and *P. verrucosum* isolated from spices samples were examined (Table 2).

56% isolates of *A. flavus* of red chilli were toxigenic and produced the highest amount of aflatoxins upto 33.6 µg/l. In turmeric, *A. parasiticus* were highly potential than *A. flavus* and also produced aflatoxins. Ochratoxin A was produced by *A. ochraceus* and *P. verrucosum* from all spice samples, but *P. verrucosum* were less potential than *A. ochraceus*. Our finding is well agreement with some other researchers [17,18]. *Penicillium citrinum* produced citrinin in red chilli and coriander but none of the *P. citrinum* of turmeric was toxic. Earlier Essono *et al* [19] also shows that 51.4% of *P. citrinum* were toxic and produces citrinin. These results show that *A. flavus* and *P. citrinum* from the red chilli and *A. ochraceus* isolated from turmeric were highly toxigenic and can produce aflatoxins, ochratoxin A and citrinin in huge amount. It may be possible that these spices enhances the virulence of *A. flavus, A. ochraceus, P. citrinum* and increases the mycotoxin producing potentiality or it may be some genetic factor. Few early reports are available regarding antifungal activity of essential oils [20].

Natural occurrence of mycotoxins in different spices

AFB_1, AFB_2, AFG_1, AFG_2, ochratoxin A and citrinin were detected in spices by LC-MS/MS (Fig. 2). During analysis, it has been observed that the spice samples were either contaminated with aflatoxin B_1 or B_1B_2 or G_1 or G_1G_2 and some were positive to $B_1B_2G_1G_2$. Ochratoxin A and citrinin were also detected from these samples (Table 3). Red chilli samples were most contaminated among all these spices. 47 samples out of 55 from red chilli were contaminated with aflatoxins among which 31samples were positive to AFB_1. Earlier, Golge *et al* [21] were also reported aflatoxins contamination in chilli from Turkey. Ochratoxin A was maximum in red chilli and only 9 samples of coriander were ochratoxin A positive. In Hungry, Fazekas *et al* [7] also reported AFB_1, AFB_2, AFG_1, AFG_2 and ochratoxin A contamination in different spices. It has been observed that citrinin contamination was only confined to red chilli and coriander samples. These samples were highly contaminated with *P. citrinum*, which are known to produce citrinin on substrate. None of the turmeric sample was citrinin positive; it may be due to inhibitory effect of essential oil of turmeric which may inhibit the growth of *P. citrinum*.

Presence of total aflatoxin, ochratoxin A and citrinin in turmeric, red chilil and coriander samples has been shown in Table 4. 85.4 % of red chilli was contaminated with total aflatoxin; level upto 219.6 ng/g followed by coriander 73.3 %. All 3 spices were contaminated with aflatoxins and the amount is maximum than ochratoxin A and citrinin in all spices (Fig. 3). Earlier Jalili and Jinap [22] have reported that 65% of chilli samples were contaminated with total aflatoxin level in the range of 0.2-79.7 ng/g and 81.25% of samples were positive to ochratoxin A in the range of 0.2-101.2 ng/g. Ozbey and Kabak [23] has also reported the aflatoxin and ochratoxin A contamination in black pepper and cumin samples. In our study, only 30 % of coriander and 57.1 % of turmeric were ochratoxin A positive. Citrinin was present in all the spices except turmeric. Ferreira *et. al*. [24] reported that turmeric have the inhibitory effect on the growth of *A. flavus* and mycotoxin production. In our report, 68.5% of turmeric was aflatoxins and 57.1% was ochratoxin A contaminated with the detectable amount of 163.8 ng/g and 125 ng/g respectively.

Table 3: Detection of different mycotoxins from spice samples.

Spices	No. of Samples Analyzed	Number of samples with different mycotoxins contamination							
		Aflatoxins						OTA[a]	CTN[b]
		B_1	G_1	B_1B_2	G_1G_2	$B_1B_2G_1G_2$	Total		
Red Chili	55	31	2	9	1	4	47	40	26
Turmeric	35	10	0	7	3	4	24	20	0
Coriander	30	15	0	4	1	2	22	9	12

[a]Ochratoxin A, [b]Citrinin

Risk assessment of isolated mycotoxins effect on human health

In spice samples aflatoxins, ochratoxin A and citrinin were detected and the amount was sufficient to induce toxicosis in human beings. The EU has provided the standard limit of mycotoxins contamination in spices (10 µg/kg for total aflatoxin and 15 µg/kg for ochratoxin A) and in present study it has been observed that the amount of mycotoxins presents naturally in the spices was much higher than the EU limit. The amount of ochratoxin A and citrinin is also sufficiently high to induce hepato-renal toxicity in humans and animals [25].

Our earlier report suggested about cumulative effects of ochratoxin A and citrinin on induction of renal carcinogenesis [2].

Conclusion

On the basis of the present study, it can be concluded that the turmeric, coriander and red chilli are susceptible substrate for fungal growth and subsequent mycotoxin productions. Aflatoxins and ochratoxin A were present in all 3 spices but citrinin were only confined to red chilli and coriander which is the first report from Bihar state. The detected amount of mycotoxins was sufficiently high to induce toxicity in human and animals. Red chilli is the most contaminated spices in which aflatoxins, ochratoxin A and citrinin was present high concentration. The present investigation will also help in the reduction of mycotoxin contamination in spices which will affect the health of consumers. So, it is very important to care in processing, handling, transportation and storage system to reduce the production of hazardous mycotoxins in turmeric, coriander and red chilli.

Table 4. Natural occurrences of Mycotoxins in the spices samples.

Mycotoxins	Spices	N.S.A[a]	Number of sample present in between different ranges							Amount (ng/g) Mean ± S.E	CV[d]	% Cont[e]
			N.D[b]	LDA[c]-100	101-200	201-300	301-400	401-500	501- ≤			
AFT[f]	R. chi	55	8	10	7	8	13	7	2	219.6 ± 21.36	0.72	85.4
	Turm	35	11	4	4	6	10	0	0	163.8 ± 25.75	0.92	68.5
	Cori	30	8	2	5	7	6	2	0	179.5 ± 27.22	0.83	73.3
OTA	R. chi	55	15	14	22	2	2	0	0	97.1 ± 12.81	0.97	72.7
	Turm	35	15	3	5	7	3	2	0	125.9 ± 24.05	1.12	57.1
	Cori	30	21	4	0	4	1	0	0	47.6 ± 17.26	1.98	30
CTN	R. chi	55	29	8	12	5	1	0	0	69.0 ± 12.50	1.34	47.2
	Turm	35	35	0	0	0	0	0	0	0	0	0
	Cori	30	18	3	2	4	2	1	0	81.0 ± 23.09	1.56	40

[a] Number of Sample analyzed, [b] Not detected, [c] Lowest detectable amount of ELISA Kit (4 ng/g for AFT, 2 ng/g for OTA, 15 ng/g for CTN), [d] Coefficient of variation, [e] percent contamination, [f]Total aflatoxins.

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Figures

Figure: 1. Fungal contamination in red chill, turmeric, coriander samples

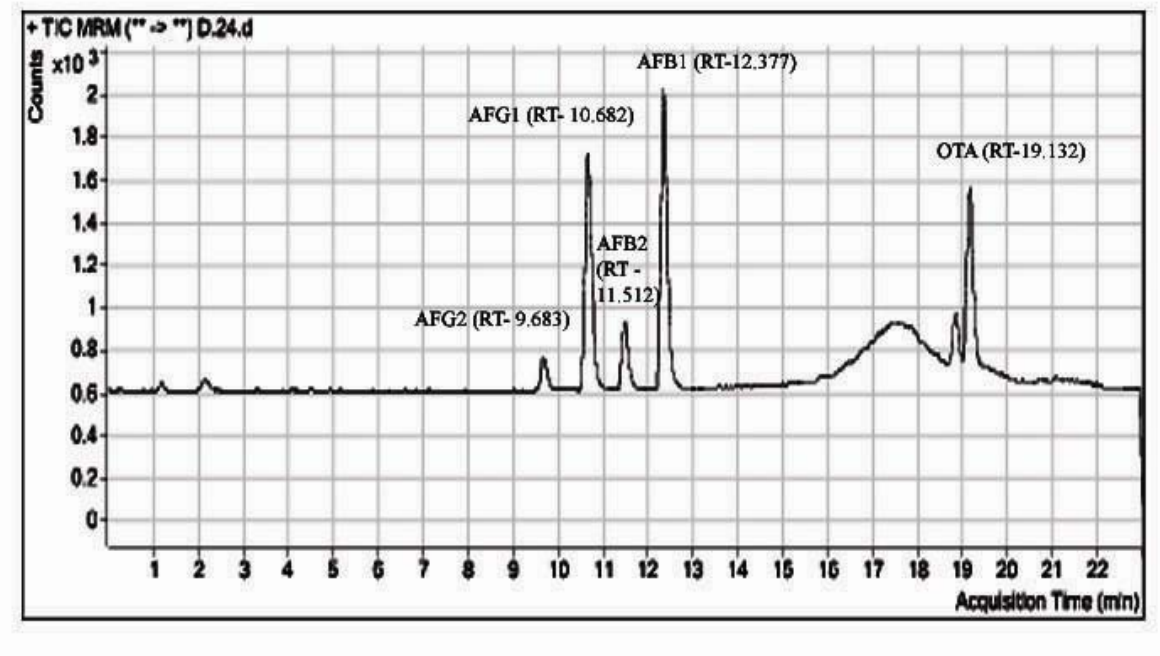

Figure 2: LC-MS/MS chromatogram of AFs (AFB_1, AFB_2,AFG_1, AFG_2) and OTA for red chilli sample having maximum contamination.

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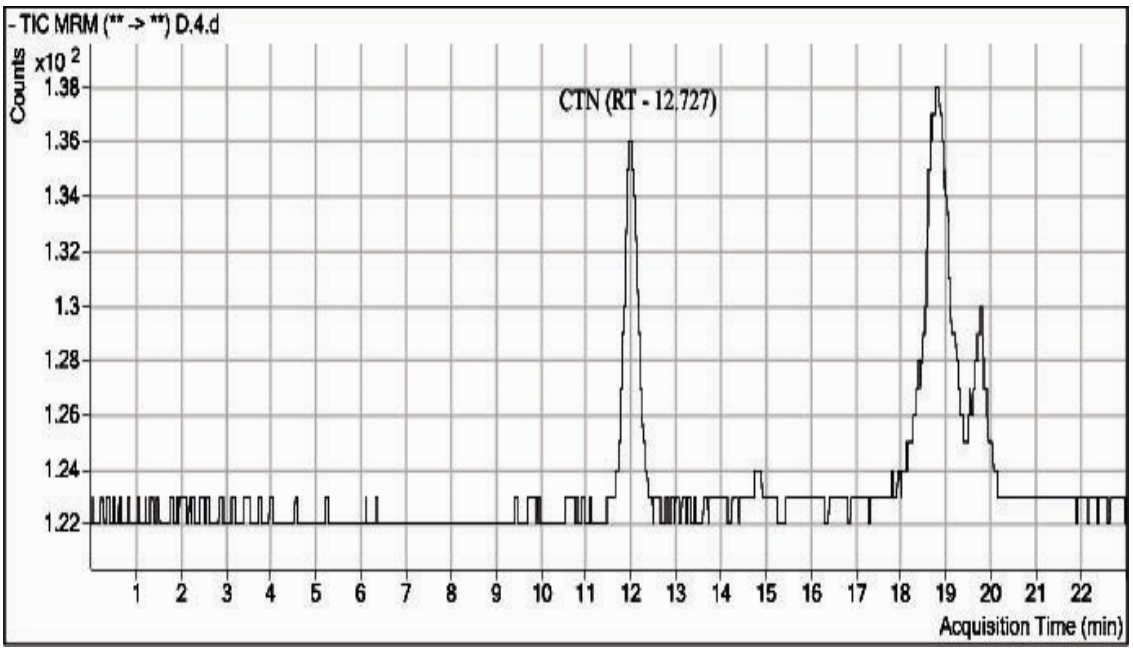

Figure 3: LC-MS/MS chromatogram of CTN for ginger sample having maximum contamination

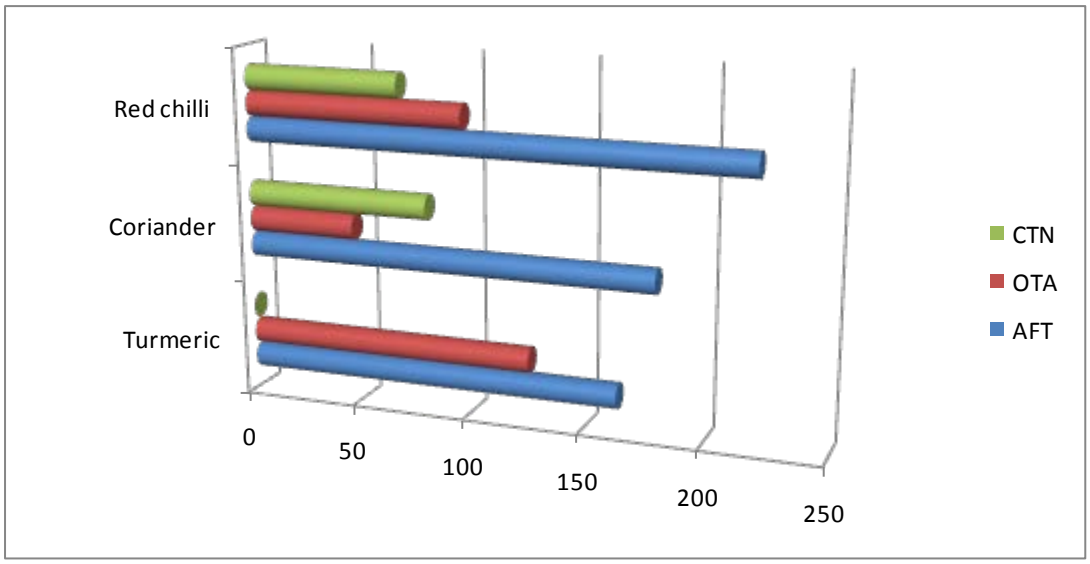

Figure 4: Red chilli shows maximum amount of asflatoxins contamination.