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Research article

Mycoflora and mycotoxins contamination in spices cultivated in Bihar (India)

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Abstract

The study was aim to determine the fungal association and aflatoxin and citrinin contamination in spices cultivated locally in Bihar (India). Red chilli, turmeric, coriander, fennel, fenugreek and dry ginger were studied for fungal and aflatoxin and citrinin contamination. *A. flavus* and *A. niger* were the most dominant species present in all types of spices. Red chilli has the highest incidence of *A. flavus* (32.3) followed by dry ginger (21.6). 56 % of *A. flavus* from red chilli were toxigenic and produced aflatoxins. Qualitative and quantitative detection of mycotoxins in spices were analyzed by Enzyme linked immunosorbent assay (ELISA). 85.4% of red chilli and 77.7% of dry ginger samples were contaminated with aflatoxins. Citrinin was present in red chilli, coriander, fenugreek and dry ginger samples. The maximum amount of aflatoxins was detected in red chilli (219.6 ng/g) and citrinin was in dry ginger (85.1 ng/g) samples. The results of this study suggest that the examined spices are susceptible substrate for growth of mycotoxigenic fungi and further mycotoxin production.

Keywords: Aflatoxins, Citrinin, Indian Spices, Toxigenic fungi, ELISA.

1. Introduction

Spices are grown all over the world but India is the largest producer of spices followed by Bangladesh, Turkey and China. About 68 different spices are cultivated in different state of India, in which Kerala, Tamil Nadu, Karnataka and Andhara Pradesh are rich in spice cultivation and production. Red chilli, turmeric, coriander, Fennel, fenugreek and ginger are important spices and also cultivated in Bihar India.

Different climatic condition of India provides the suitable environment for the cultivation of spices. The Temperature varies from 20°C to 28°C which 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]. Citrinin is generally produced by *P. citrinum*, *A. ochraceus* and *A. terreus* and it is nephrotoxic and also affects the immune system in animals[2,3].

Some fragmentary reports are available regarding mycofloral and mycotoxins contamination in spices from India and different part of the world. However these reports are mainly confined to aflatoxins and ochratoxin A contamination in spices [4-6].

The present study was conducted to ascertain the predominant mycoflora associated with spices, and there mycotoxin producing potentiality and occurrence of aflatoxins and citrinin in the spice samples cultivated in Bihar. During the investigation it has been observed that the examined spices are susceptible to aflatoxins and citrinin contamination. Citrinin was also present in some spice samples in extremely high amount. The amount of aflatoxins and citrinin present in spices were sufficiently high to induce toxicity in the consumers and also due to presence of high amount of amount of mycotoxins contamination, they can't be exported to many countries because it is much higher than the permissible limit.

2. Material and methods

2.1. Sampling

25 samples of each spices (red chilli, turmeric, coriander, fennel, fenugreek and dry ginger, total 150 samples belonging to 6 types of spices were collected from highly cultivated area of Bihar.

2.2. Isolation and Identification of fungi

All the samples had randomly placed on the freshly prepared Potato dextrose agar (PDA) and on 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 [7] for genus *Aspergillus*, Pitt [8] for *Penicillium*, Leslic & Summerell [9]for *Fusarium* and Crous et al. (2009) for other genera.

2.3. Analysis for Potentiality of mycotoxins producing isolated fungi

Mycotoxins producing potentiality of *A. flavus*, *A. parasiticus*, *A. terreus*, *P. citrinum* and *P. verrucosum* were examined. The suspensions of isolated fungi were prepared by Macfarland standard in normal saline that each ml of saline contains 10^6 spores. In all cases 50µl of each suspension was inoculated in 25ml of freshly prepared broth media (SMKY media for aflatoxins and YES media for citrinin) and incubated at 28 ± 2°C for 10 days. When vigorous growth of fungus occurred the medium was filtered with Watman No.1 paper and the cultured filtrate was extracted with 10 ml of 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 and qualitative and quantitative estimation of mycotoxins producing potentiality of fungi were done by the method of Diener & Davis [11] for aflatoxins producing potentiality of *Aspergillus* species. Methods of Schwenk et al. [12] was followed for citrinin producing potentiality of mycoflora respectively.

2.5. Qualitative and quantitative estimation of natural occurrence of mycotoxins by ELISA

Natural occurrence of aflatoxins and citrinin in spices samples were analyzed by enzyme linked immunosorbent assay (ELISA). Samples were examined by AgraQuant Total Aflatoxin (COKAQ1000) for aflatoxins from ROMER LAB (ASTRIA) and RIDASCREEN FAST citrinin Assay (6302) for citrinin. For qualitative and quantitative estimation of aflatoxins and citrinin, 20 gm of sample were grinded and added 100 ml of 70% methanol blended for 3 minute. The solutions were filtered and the supernatant was collected. 4ml of extract was transferred through cleanup columns then the amount of aflatoxins and citrinin were detected with specific ELISA kits and the optical density was recorded by the ELISA reader using a 450 nm filter with a differential filter of 630 nm. The minimum detected amount of ELISA kit was 4 ng/g for aflatoxins and 15 ng/g for citrinin. Standard curve was prepared with standard solution provided with ELISA kits. The optical densities of the samples were compared to the optical density of standards and interpretative results were determined. Further natural occurrence of these mycotoxins was also assessed by using Thomas et. al. [13] method for aflatoxins and Robert & Paterson [14] method for rest of mycotoxins using TLC.

3. Result and discussion

3.1. Mycofloral association with spices

A large numbers of fungal species were isolated from examined spices. A total of 7 different fungal genera belong to 22 species were isolated (Table 1). *Aspergillus* was isolated from all spice samples whereas *Penicillium* were confined only to red chilli, turmeric, coriander, fenugreek and dry ginger samples. Elshafie et. al. [15] also reported these fungi from ginger and coriander. Percent incidence of toxigenic mycofloral contamination is presented in Fig. 1 and it shows that *A. flavus* contamination was highest in red chilli (32.3) followed by dry ginger (21.6) and lowest was in coriander (8.0) samples. The present study revealed the wide range of fungal contamination in spices in which *A. flavus* and *A. niger* were the most dominant and present in all 6 types of spice samples; the present finding supports the report of Bokari [16]. Moreover, the result from table 1 also shows that some of the fungi were only confined to specific spices. *A. alternata, A. tamari* and *C. globosum* species were only confined to red chilli samples whereas *P. citrinum* and *A. ochraceus* were present in all spices except in fennel and coriander respectively. It has been observed that red chilli and dry ginger are susceptible substrate for growth of *A. niger, A. flavus, A. ochraceus, P. citrinum, P. verrucosum, F. moniliforme* and *R. oryzae* (Fig 2) but coriander, turmeric, fennel and fenugreek are not suitable for the growth of *Alternaria alternata, A. tamarii, A. versicolor, P. purpurogenum* and *Chaetomium globosum* mycoflora.

3.2. Mycotoxin producing potentiality of isolated fungi

A. flavus, A. parasiticus, A. terreus, P. citrinum and *P. verrucosum* isolated from spices samples were examined for mycotoxin producing potentiality (Table 2). Toxigenic *A. flavus* were detected from all samples of spices and about 56% of *A. flavus* from red chilli samples were found toxigenic. *A. parasiticus* also produced aflatoxins in dry ginger but less potential than *A. flavus*. *P. citrinum* produced citrinin in red chilli, coriander, fenugreek and dry ginger samples with potentiality upto 28.3 µg/l , 20.9 µg/l , 19.2.5 µg/l ,22.3 µg/l . None of the *P. citrinum* of turmeric was toxic. Similar finding were reported earlier by Essono et al. [17] that *P. citrinum* shows 51.4% of toxicity and produces citrinin with the level ranges from 0.8-3.7 µg/l. In our investigation, *P. verrucosum* also produce citrinin whereas none of the isolates of *A. terreus* were found toxigenic. Our finding is well agreement with some other researchers [18,19].These results show that *A. flavus* isolated from the red chilli and *P. citrinum* from dry ginger were highly toxigenic and can produce aflatoxins and citrinin in high amount. So, it may be possible that these spices enhances the virulence of *A. flavus, P. citrinum* and increases the mycotoxin producing potentiality. This is the matter of further research.

3.3. Natural occurrence of mycotoxins in different spices samples.

Presence of aflatoxins and citrinin in different concentration in 6 different spices has been shown in table 3. During the analysis, 85.4% of red chilli samples were positive to aflatoxins followed by dry ginger (77.7%) whereas fennel (56%) has the lowest contamination. Highest amount of aflatoxins were recorded in red chilli samples (219.6 ng/g) and the lowest concentration of aflatoxins was recorded in fennel (84.1 ng/g). All samples of spices were contaminated with aflatoxins and the amount of aflatoxins is maximum than citrinin except in fennel (Fig. 3). Earlier Jalili & Jinap [20] has also reported that 65% of chilli samples were contaminated with aflatoxins level in the range of 0.2-79.7 ng/g. The present investigation also recorded that 47.2% of red chilli, 40% coriander, 37.1% fenugreek and 44.4% of dry ginger samples were contaminated with citrinin and the amounts were 69.0 ng/g, 81.0 ng/g, 63.1ng/g and 85.1ng/g respectively and none of the samples of turmeric and fennel were positive to citrinin.

3.4. Risk assessment of isolated mycotoxins on human health

In spice samples aflatoxins 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 aflatoxins) and we observed that the amount of mycotoxins presents naturally in the spices were much higher than the EU limit. The amount of aflatoxin and citrinin is also sufficiently high to induce carcinogenesis and renal toxicity in human and animal [3,21].

4. Conclusion

On the basis of the present study, it may be concluded that the examined spices are susceptible substrate for toxigenic fungal growth and aflatoxins production and citrinin production except in turmeric and fennel. The detected amount of aflatoxins and citrinin was sufficiently high to induce carcinogenicity and renal toxicity in human and animals. Red chilli and dry ginger are the most contaminated spices in which toxigenic fungi and mycotoxins were present in high concentration whereas Fennel and turmeric are the spices which can be considered a bit resistant to mycotoxigenic fungi and mycotoxin contamination. The present investigation will also help in the reduction of mycotoxin contamination in spices which will affect the Indian economy because India produces about 75 % of the total spices of the world but only 40 % were exported because the contamination level is extremely higher than the permissible limit of mycotoxin contamination in many countries. So, it is very important to care in processing, handling, transportation and storage system to reduce the production of hazardous mycotoxins.

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Table 1. Percent incidence of fungi isolated from different spices samples.

Fungal species	Name of the spices					
	R. chi	**Turm**	**Cori**	**Fenn**	**Fenu**	**D. ging**
Alternaria alternata	2.3	-	-	-	-	-
Aspergillus parasiticus	-	-	-	-	-	2.0
Aspergillus oryzae	-	-	-	-	-	1.3
Aspergillus tamarii	5.3	-	-	-	-	-
Aspergillus niger	15.3	8.0	10.6	5.3	2.6	9.0
Aspergillus flavus	32.3	9.6	8.0	12.3	19.6	21.6
Aspergillus ochraceus	8.6	7.6	-	5.0	2.3	4.6
Aspergillus versicolor	11.42	-	-	-	-	-
Aspergillus fumigatus	-	-	2.0	-	-	4.6
Aspergillus terreus	-	-	-	1.3	-	2.6
Aspergillus sydowi	-	-	2.6	-	2.6	-
Penicillium citrinum	12.6	4.00	7.6	-	7.6	16.3
Penicillium islandicum	-	-	-	-	-	-
Penicillium verrucosum	8.6	8.0	6.3	-	6.0	8.6
Penicillium purpurogenum	-	-	-	-	-	2.6
Penicillium cyclopium	-	-	-	-	-	-
Fusarium oxysporum	-	5.3	-	-	-	2.3
Fusarium moniliforme	6.3	4.6	-	3.0	1.6	3.0
Chaetomium globosum	1.40	-	-	-	-	-
Rhizopus nigricans	-	-	4.3	-	-	-
Rhizopus oryzae	4.3	-	-	2.3	4.0	2.6
Mucor hiemalis	7.0	-	2.6	1.6	-	-

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

Mycotoxins	Samples	% Contamination	Amount (ng/g) Mean ± S.E	Coefficient of variation
Aflatoxins	Red chilli	85.4	219.6 ± 21.36	0.72
	Turmeric	68.5	163.8 ± 25.75	0.92
	Coriander	73.3	179.5 ± 27.22	0.83
	Fennel	56	84.1 ± 20.25	1.20
	Fenugreek	65.7	135.4 ± 24.85	1.08
	Dry ginger	77.7	183.6 ± 25.04	0.81
Citrinin	Red chilli	47.2	69.0 ± 12.50	1.34
	Turmeric	0	0	0
	Coriander	40	81.0 ± 23.09	1.56
	Fennel	0	0	0
	Fenugreek	37.1	63.1 ± 17.25	1.61
	Dry ginger	44.4	85.1 ± 19.46	1.37

Table 3. Natural occurrences of Mycotoxins in the spices samples.

S. No.	Sample	Fungi examined	Positive/N.I.A[j]	% toxicity	Mycotoxin detected	Potential range (µg/l)
1.	R. chi	*Aspergillus flavus*	14/25	56.0	Aflatoxins	6.8 – 33.6
		Penicillium citrinum	5/16	31.2	Citrinin	18.4 – 28.3
		Penicillium verrucosum	4/10	40.0	Citrinin	5.5 – 8.6
2.	Turm	*Aspergillus flavus*	4/20	20.0	Aflatoxins	7.4 – 11.3
		Penicillium citrinum	0/15	0	-	-
		Penicillium verrucosum	3/12	25.0	Citrinin	5.7 – 6.1
3.	Cori	*Aspergillus flavus*	7/18	38.8	Aflatoxins	7.2 – 13.7
		Penicillium citrinum	4/15	26.6	Citrinin	18.7 – 20.9
		Penicillium verrucosum	3/15	20.0	Citrinin	9.8 – 16.1
4.	Fenn	*Aspergillus flavus*	9/20	45.0	Aflatoxins	16.2 – 24.3
		Aspergillus terreus	0/15	0	-	-
5.	Fenu	*Aspergillus flavus*	7/20	35.0	Aflatoxins	9.1 – 19.7
		Penicillium citrinum	3/15	20	Citrinin	18.6 – 19.2
		Penicillium verrucosum	0/15	0	-	-
6.	D. Ging	*Aspergillus flavus*	11/25	44.0	Aflatoxins	10.7 – 24.2
		Aspergillus parasiticus	5/18	27.7	Aflatoxins	7.4 – 8.1
		Aspergillus terreus	0/15	0	-	-
		Penicillium citrinum	7/18	38.8	Citrinin	18.5 – 22.3
		Penicillium verrucosum	3/10	30.0	Citrinin	5.4 – 8.7

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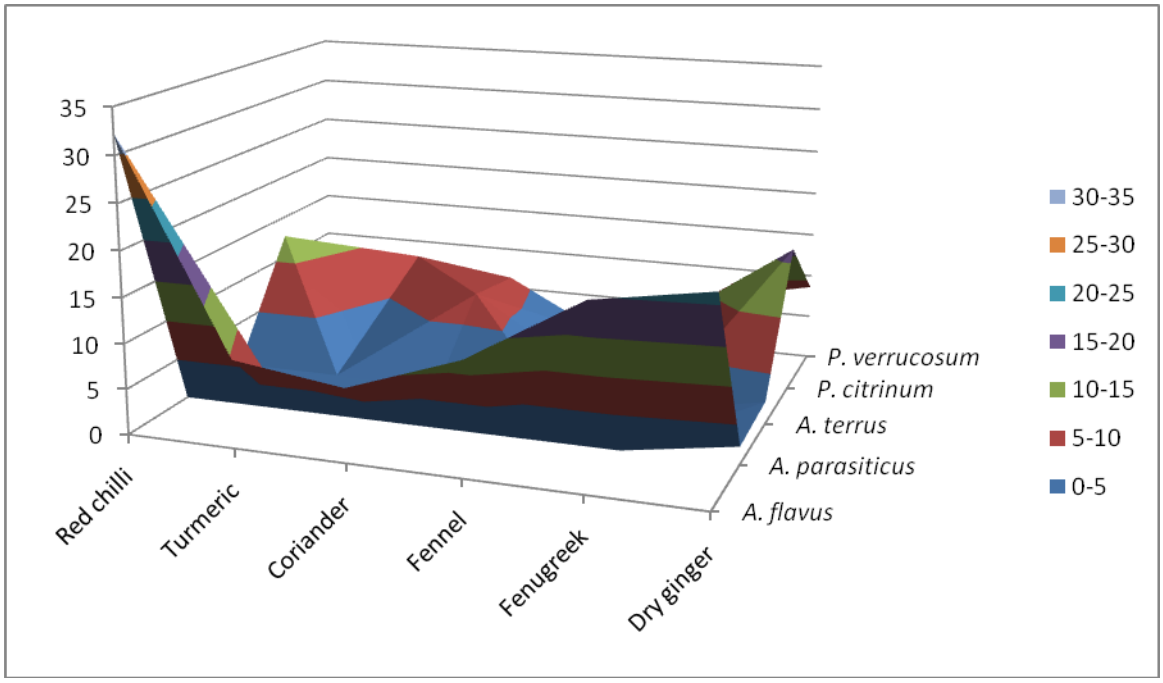

Figure 1. Percent incidence of toxigenic fungi isolated from different spice sample

Figure 2. *Fusarium* and *Aspergillus* association in red chilli samples.

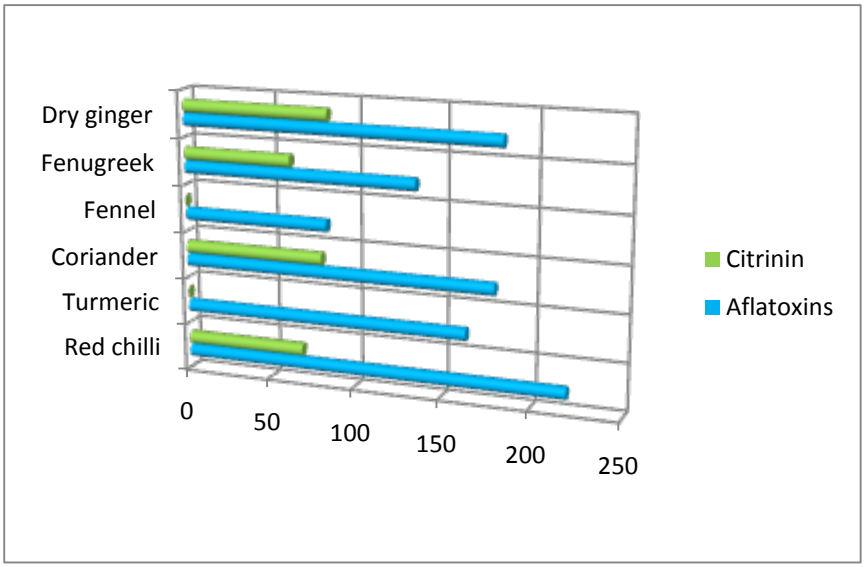

Figure 3. Different amount of aflatoxins and citrinin detected from spices