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Quantify the mycotoxins produced by the important fungi

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Abstract

Spices can be contaminated with various hazards, among which toxigenic fungi are probably the most important. Indeed, some fungal species produce toxic secondary metabolites named mycotoxins as they develop on human food and animal feed. Among the hundreds of known mycotoxins, aflatoxins are the major ones for public health because they are the most potent of the known natural carcinogens.

Keywords: Mycotoxins, fungi, Spice, crops, Microbiology

Introduction

Aflatoxins are produced by different fungal species that belong to the genus *Aspergillus* and more specifically to the Flavi section. For years, three main aflatoxigenic species were commonly considered in the section Flavi: *A. flavus*, *A. parasiticus* and *A. nomius*. In the last decade, the use of molecular tools enabled the identification of new species belonging to the section Flavi, comprising 34 different species, of which 17 are aflatoxigenic. These species can be distinguished by subtle morphological specificities, molecular changes in some gene sequences and, most importantly, through their ability to produce different mycotoxins. Indeed, some species, including *A. flavus, A. pseudotamarii* and *A. togoensis*, produce aflatoxins of B type, whereas others, including *A. parasiticus*, *A. minisclerotigenes*, *A. mottae*, *A. nomius*, *A. novoparasiticus*, *A. arachidicola* and *A. korhogoensis* produce both B and G type aflatoxins. Some species may also produce other toxic secondary metabolites such as cyclopiazonic acid.

Many spice crops and their products are susceptible to fungal attack either in the field or during storage. Some of these fungal species can produce as secondary metabolites a diverse group of chemical substances known as mycotoxins. The three important genera associated with mycotoxin production are: *Aspergillus*, *Penicillium* and *Fusarium*. Mycotoxins have been reported to be carcinogenic, teratogenic, tremorogenic, haemorrhagic, and dermatitic to a wide range of organisms and to cause hepatic carcinoma in man. When these contaminated spices are used in food, it will harm the health of the consumers. Mycotoxins are the secondary metabolites of fungi, which are toxic and cause diseases and organ disorder in animals as well as in humans. Aflatoxins are toxic, mutagenic, carcinogenic and immunosuppressive agents and are produced by *Aspergillus flavus and A. parasiticus* on a variety of food products. Among 18 different types of aflatoxins, aflatoxin B1, B2, G1 and G2 are major mycotoxins present in food and food products in which Aflatoxin B1 (AFB1) is predominant. Ochratoxin A is anticipated to be a potent nephrotoxin causing kidney damage

Materials and Methods

Sample collection: The present study was undertaken to isolate and identify the fungi contaminating the various spices, being sold in Central Indian city of Jabalpur, both in loose as well as packed conditions. In order to study the status of fungal contaminations in various spices, major spices used in Indian culinary were chosen. Ground spice samples were procured from the local market, including supermarkets and warehouses. The major spices chosen were red chilli powder, coriander powder, cinnamon powder, turmeric powder, black pepper powder and curry powder (Garam masala).

Isolation of fungi associated with spice samples

Fungi associated with spices were isolated by direct plating

technique. The dilutions of the samples were first prepared in sterile water. Dilutions of 10^{-1} and 10^{-2}were prepared and 1 ml of both the dilutions was inoculated onto potato dextrose agar (PDA) medium containing 0.25% chloramphenicol as antibacterial agent.

Identification of fungi

The fungi were identified according to their macro- and microscopic features. Cultures were examined periodically and identified during the sporulation phase. The cultures were divided into groups based on their morphological characteristics including growth pattern, colony texture, pigmentation, and growth rate of the colonies on PDA (Promputtha *et al.*, 2005) [4]. When fungal colonies sporulated on PDA, small plaques from the edge and the centre of each growing colony were transferred onto glass slides, and then were examined using a compound light microscope (Labomed, India) for characteristics of their vegetative and reproductive structures such as hyphal colour and structures, shape and size of conidia and conidiophores. The cultures were stained with lactophenol cotton blue.

Analysis of Mycotoxins

The fungi, isolated from spice samples were screened for their ability to produce mycotoxins, mainly aflatoxins. For this, the isolated fungi were mass cultivated in potato dextrose broth for 7 days, with continuous agitation at low speed. After 7 days, the cultures were filtered, and the cell free filtrate of the cultures was collected in a Cryo bottles and kept at -20 °C till further use.

Aflatoxin Standards

The certified reference material for aflatoxins, containing AFB1, AFB2, AFG1 and AFG2 was obtained from Sigma Aldrich, USA. All reagents for the mobile phase used were HPLC grade from Merck-Millipore Ltd. (Germany). The solid-phase extraction (SPE) used a LiChrolut C-18 Cartridge from Merck-Millipore, Germany (6 ml volume).
Aflatoxin certified reference material was diluted to 10 mL usingacetonitrile to make a stock solution that was serially diluted into 1 ppm working solutions.

Sample preparation

For sample analysis, the sample prepared for TLC, as shown above was used. The sample was further processed using an immuno- affinity clean up column (Aflatest, Sigma, USA). The column was loaded with the samples and washed with the distilled water twice. The aflatoxins were eluted using 1 ml of methanol, and diluted with 1 ml of distilled water. The sample was filtered using a 0.22 µm syringe filter (Thermo Fisher, USA), and placed in HPLC sample vials for injection. The samples were injected using an automated program to the system. A fraction of 10 µL was injected for each run.

HPLC run

The mobile phase consisted of 63:26:11 H_2O: Methanol: Acetonitrile, adjusted to pH 6.8 with glacial acetic acid, and was filtered through a 0.45 µm membrane filter (Whatman, USA) under vacuum.

Results

The isolated fungi were identified using macroscopic and microscopic characters based on the available literature and taxonomic keys. In all, 13 fungal strains could be isolated. Fig 1 shows the macroscopic characters of fungal colonies appearing on to the PDA plate. The front and back view of the fungal colonies is shown. Microscopic characters of isolated fungi are Among the isolated fungi were: *Aspergillus flavus*, *Fusarium sp*., *Fusarium oxysporum*, *Penicillium sp*., *Mucor racemosus*, *Aspergillus niger*, *Aspergillus terreus*, *Rhizopus sp*., *Alternaria sp*., *Aspergillus parasiticus*, *Aspergillus fumigatus*, *Cladosporium sp*. and *Curvularia sp*.When the dual culture experiments were conducted with Aspergillus parasiticus as target fungus, similar results were observed with different test fungi.That while the test fungus alone achieved radial mycelia growth of 86 mm in 7 days, it was reduced up to 72% with test fungi. All the test fungi were able to inhibit the growth of *A. parasiticus* almost up to the same level and the radial mycelia growth was reduced up to 24-27 mm after 7 days of the *in vitro* growth. The reduction in mycelia growth can well be observed as with Mucor racemosus, with *A. niger*, with *A. terreus*, with *Rhizopus oryzae*, with *Alternaria alternata*with *Cladosporium herbarum*, with *Curvularia lunata*, with *Penicillium chrysogenum* and *Fusarium equiseti*.

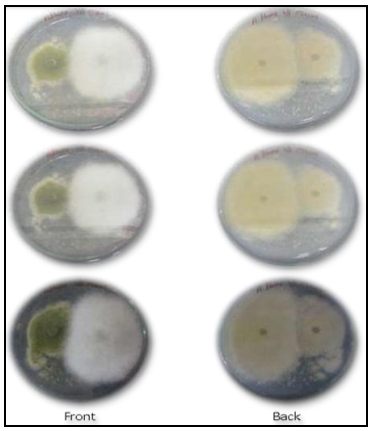


Fig 1: Antagonistic activity of *A. flavus* aaingt *Mucor racemosus*

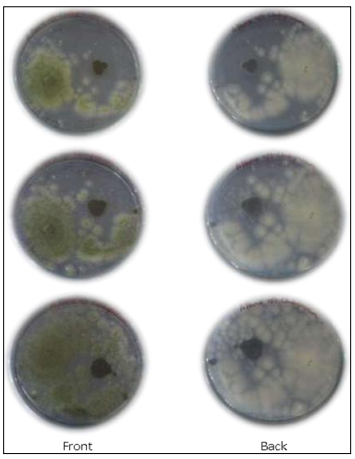


Fig 2: Antagonistic activity of *A. flavus* against *Cladosporium herbarum*.

Table 1: Incidences of fungal infestations in different Indian spices due to Aspergillus genus with emphasis on Aspergillus section Flavi members from the spice samples collected from Central India

S. No.	Type of spice	No. of samples collected	Number of samples with fungal infestation	Samples having presence of *Aspergillus* species	Presence of member of *Aspergillus* section *Flavi*	
					A. flavus	*A. parasiticus*
1.	Bay Leaves	7	7	4	1	0
2.	Black cumin	4	4	4	1	0
3.	Black pepper	4	4	2	0	0
4.	Cinnamon	10	10	10	4	1
5.	Clove	10	9	6	3	0
6.	Coriander	19	18	31	11	4
7.	Cumin seeds	5	5	4	1	0
8.	Fenugreek	4	3	4	0	0
9.	Curry Powder	11	11	6	2	0
10.	Red Chilli	15	12	16	5	0
11.	Turmeric	12	10	13	3	1
	Total	101	93	100	31	6

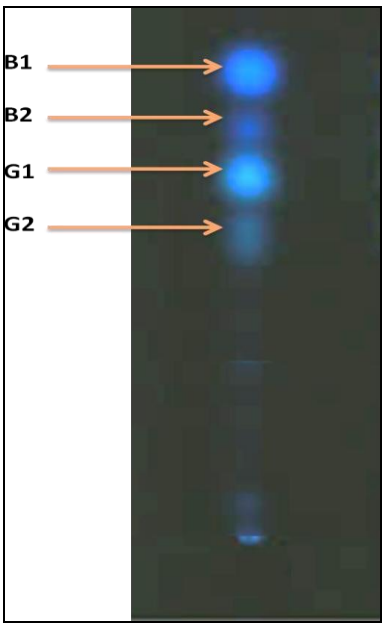


Fig 3: Standard aflatoxin mixture chromatographed on to Thin layer chromatographic plate showing the bands of four aflatoxins.

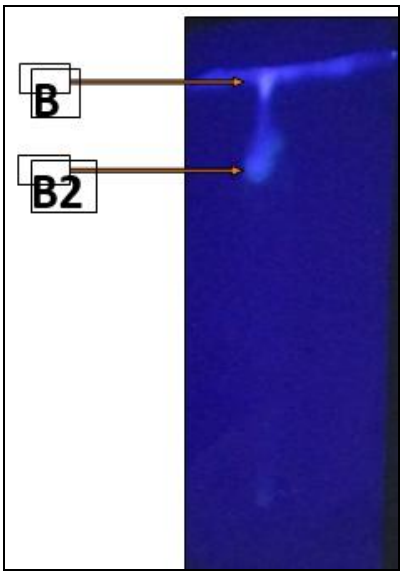
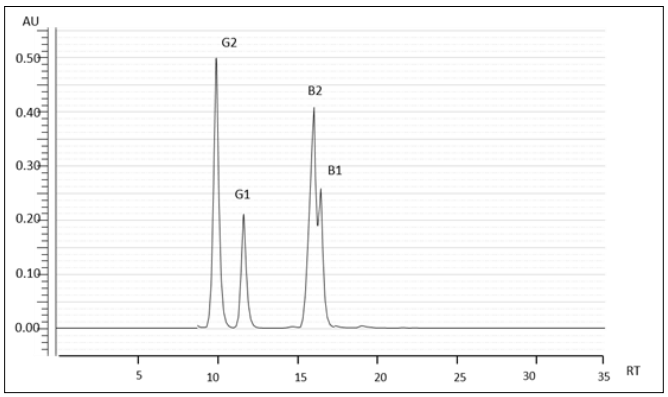


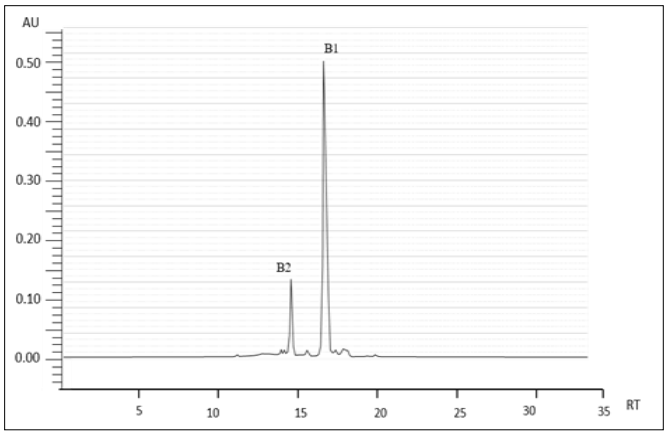
Fig 4: Thin Layer Chromatography of samples of Garam masala sample 8 (01.06.22). The fluorescence under the UV light shows the presence of aflatoxins, which were then correlated to the standard aflatoxin mixture for identification.



Peak Results

No.	Peak Name	Retention Time min	Area mV*min	Height AU	Relative Area %	Relative Height %	Amount %	Amount µg /g-1
1.	Aflatoxin G2	10.02	19.154	0.50	0.50	10.37	**10.653**	105.52
2.	Aflatoxin G1	11.23	51.248	0.21	0.21	37.21	**24.885**	282.34
3.	Aflatoxin B2	15.87	21.268	0.41	0.41	15.44	**9.5947**	117.17
4.	Aflatoxin B1	16.21	50.931	0.26	0.26	36.98	**25.0323**	280.59

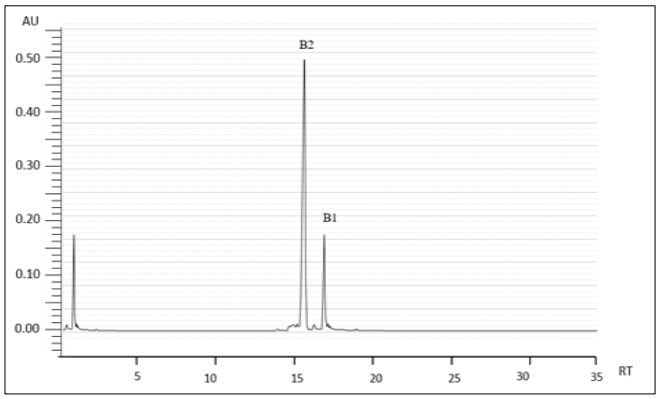
Fig 5: HPLC chromatogram of aflatoxin standards showing retention times of four aflatoxins.



Peak Results

No.	Peak Name	Retention Time min	Area mV*min	Height AU	Relative Area %	Amount %	Amount µg /g-1
1.	Aflatoxin G2	NA	NA	NA	NA	NA	NA
2.	Aflatoxin G1	NA	NA	NA	NA	NA	NA
3.	Aflatoxin B2	15.1	6.224	0.12	4.52	8.57	34.29
4.	Aflatoxin B1	16.3	97.94	0.50	71.11	134.89	539.80

Fig 6: HPLC chromatogram of extracted coriander sample



Peak Results							
No.	Peak Name	Retention Time min	Area mV*min	Height AU	Relative Area %	Amount %	Amount µg /g-1
1.	Aflatoxin G2	NA	NA	NA	NA	NA	NA
2.	Aflatoxin G1	NA	NA	NA	NA	NA	NA
3.	Aflatoxin B2	NA	NA	NA	NA	NA	NA
4.	Aflatoxin B1	NA	NA	NA	NA	NA	NA

Fig 7: HPLC chromatogram of extracted bay leaves sample

Conclusion

Although there are more than 300 mycotoxins discovered so far, only few are known to infest food commodities. Spices are reported to have only aflatoxin contaminations, and hence FSSAI has set up the limit of 30 ppm for spices (www.fssai.gov.in). Although this limit is almost twice as the limit of European Union, it is important to note that most of the spices are cultivated in tropical conditions, including India, where knowledge about toxin contamination and technological advantage for storage and transport is negligible. Hence, these crops are more prone to fungal infestations, and in turn, aflatoxin contaminations.

Our study concluded that

1. Almost 95% of total spice samples were infested with fungi, and in most cases with more than 2 fungal species
2. About 20% of spice samples of Jabalpur area are contaminated with toxigenic fungi
3. AFB1 is the most dominant aflatoxin found in spices followed by AFB2.
4. Coriander was the most infected spice in Jabalpur.
5. *Aspergillus flavus* and *A. parasiticus* were the two toxigenic strains isolated from the spices.
6. Fungi isolated from spice samples, but not found to produce aflatoxins, suitably reduced the growth of *A. flavus* and *A. parasiticus* under *in vitro* conditions. The radial mycelial growth was reduced in a magnitude of 70%.

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