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Natural occurrence of toxigenic mycoflora and ochratoxin A & aflatoxins in commonly used spices from Bihar state (India).

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Abstract: *The present study was carried out to investigate the aflatoxigenic and ochratoxigenic fungi and their mycotoxins from 194 samples of 5 types of spices (black pepper, turmeric, fennel, green cardamom and mace). Ochratoxin A producing A. niger and aflatoxin producing A. flavus were the most dominant species present in all types of spice. 52% of A. flavus and 44% of A. parasiticus from black pepper were toxigenic and produce aflatoxins where as 40% of A. ochraceus form black pepper and 33.3% of P. verrucosum from green cardamom were produce ochratoxin A. Qualitative and quantitative detection of mycotoxins in spices were analyzed by thin layer chromatography (TLC) and Enzyme linked immunosorbent assay (ELISA). 78.1% of black pepper samples were contaminated with aflatoxins followed by mace (63.3%). Both aflatoxins and ochratoxin A were present in all types of spices. The maximum amount of aflatoxins and ochratoxin A was in black pepper 320ppb and 155ppb respectively. The result of this study suggests that the spices are rich substrate for growth of ochratoxigenic and aflatoxigenic fungi and further ochratoxin A and aflatoxins production. The amounts detected in these spices were sufficiently high to induce carcinogenesis. This is the first report of occurrence of ochratoxin A in spices from Bihar state (India).*

Keywords: *Toxigenic fungi; Spices; Aflatoxins; Ochratoxin A; ELISA.*

I. Introduction

Worldwide, spices are used to provide distinct color and aroma to foods and have also the medicinal properties. India is the largest spice producer country in the world and more than 80 types of spices are cultivated here. Black pepper, turmeric, fennel, green cardamom and mace are such spices which are commonly used in Bihari cuisine. Spices are generally grown in tropical climate which is favorable for the microbial growth and mycotoxins production and poor processing and sanitation of these spices enhances the contamination. During storage these contaminants increased and produced mycotoxins in extremely high amount [1]. When these contaminated spices were used in food, it will harm the health of consumers. Mycotoxins are the secondary metabolites of fungi which are toxic and cause diseases and organ disorder in animals as well as in humans [2, 3]. Aflatoxins are toxic, mutagenic, carcinogenic and immunosuppressive agents, produced as secondary metabolites by the fungus *A. flavus* and *A. parasiticus* on variety of food products. Among 18 different types of aflatoxins, aflatoxin B_1, B_2, G_1 and G_2 are major mycotoxins present in food and food products in which Aflatoxin B_1 (AFB_1) is normally predominant in amount. Ochratoxin A is reasonably anticipated to be a potent nephrotoxin in animals as well as in humans. OTA causes kidney damage, is based on sufficient evidence of carcinogenicity from experimental animals and the studies also suggest the relation of OTA exposure and a fatal human kidney disease called Balkan endemic nephropathy [4, 5].

Few reports are available regarding aflatoxins and ochratoxin A contamination in some spices in the different part of the world [6]. But this is the first report of occurrence of ochratoxin A in spices from Bihar state (India). Earlier, only aflatoxin contamination was reported in medicinal herbs from Bihar [7]. In our investigation it has been observed that the amount of aflatoxins and ochratoxin A present in spices is much higher than the permissible limit.

The present study was conducted to ascertain the predominant mycoflora especially aflatoxigenic and ochratoxigenic mycoflora associated with spices, and their mycotoxin producing potentiality. During the investigation it has been observed that the examined spices are susceptible to aflatoxins and ochratoxin A contamination. The presence of these mycotoxins in these spices is alarming and a matter of concern because these mycotoxins can directly affect the human health and are well known for their carcinogenic effect.

II. Material And Methods

2.1 Sampling

55 samples of black pepper, 42 turmeric, 32 fennel, 35 green cardamom and 30 mace samples, total 194 samples were collected from local market of Patna, Bihar. Each sample was put into the sterile cellophane bag and then put into the sterile brown envelop and stored at 4°C to arrest any mycotoxin formation before analysis.

2.2 Isolation and Identification of fungi

All the samples were randomly placed on PDA media & standard blotter paper and incubated at 28 ± 2°C for 5- 7 days and examined daily. Micro-organism were isolated by hyphal tip method and examined visually and by binocular stereomicroscope. Identification was carried out by morphological characteristics and followed the taxonomic schemes of Maren [8] for genus *Aspergillus*, Pitt [9] for *Penicillium,* Nelson [10] for *Fusarium* and Funder [11] for other genera.

2.3 Screening of fungi for their mycotoxin producing potentiality

Isolated mycoflora were analyzed for mycotoxins production and there potentiality. Aflatoxin producing potentiality of *Aspergillus parasiticus* & *A. flavus* and ochratoxin A producing potentiality of *A. niger, A. ochraceus* and *P. verrucosum* were examined. The suspensions of isolated mycoflora were prepared using Mcfarland that each ml of saline contains 10^6 spores [12]. In all cases 50 µl of each suspension was inoculated in 25 ml of freshly prepared broth media 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. 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 Dienern [13] for aflatoxins producing potentiality of Aspergillus spp. and Davis [14] for testing OTA producing potentiality of *A. niger, A. ochraceus* and *P. verrucosum.*

2.4 Qualitative study of mycotoxin in samples

The samples were analyzed for the mycotoxins contamination by TLC method. 50 gm of each sample were powdered and blended with 250 ml of methanol: water (60:40) for 15 min at high speed. The methanolic extract was filtered through watman no.1 filter paper. 125 ml of filtrate was taken in 500 ml separating funnel and add 30ml of saturated NaCl and 50 ml of n-Hexane, and shaken vigorously for 5 min. The lower methonilic layer was collected in another separating funnel and added 40 ml of chloroform and shaken again for 5 min. The lower chloroform layer was obtained in flask containing 5gm of cupric carbonate and agitated then the chloroform was decanted through the bed of Na_2So_4 with watman no. 42 filter paper. The chloroform extract was evaporated to dryness and residue was dissolved in 1ml of chloroform. 50 µl of chloroform extract of each samples were placed on TLC plated along with the mycotoxins standards. For aflatoxins, toluene: Isoamyl alcohol: methanol (90:32:2 v/v/v) was used [15] and for the detection of ochratoxin A Benzene: methanol: acetic acid (24:2:1 v/v/v) was used [16]. The dry developed plate was observed in UV light under long and short wavelength.

2.5 Quantitative study of mycotoxins in samples by ELISA

The quantitative detection for natural occurrence of ochratoxin A and aflatoxin in samples were analyzed by enzyme linked immunosorbent assay (ELISA) [17]. Samples were analyzed by AgraQuant Total Aflatoxin (COKAQ1000) for aflatoxin and AgraQuant Ochratoxin (COKAQ2000) for ochratoxin A from ROMER LAB (ASTRIA).20 gm of each 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 presence of ochratoxin and aflatoxin 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. ELISA kit has maximum limit of 40 ppb, so the samples were diluted. 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 using dilution factor.

III. Result and discussion

3.1 Natural occurrence of toxigenic fungi

The prevalence of mycoflora was observed in associated with spices (Table 1), in which some of them were toxigenic in nature and produced different mycotoxins. These fungi were *Aspergillus parasiticus, A. oryzae, A. niger, A. flavus, A. ochraceus, A. versicolor, A. terreus, Penicillium citrinum, P. verrucosum, P. purpurogenum, Fusarium oxysporum, F. moniliforme, Rhizopus nigricans, R. oryzae* and *Mucor hiemalis*. In which *Aspergillus parasiticus* and *A. flavus* are aflatoxigenic fungi and *A. niger, A. ochraceus* and *P. verrucosum* are ochratoxigenic fungi. In our study, *A flavus* was most dominated and present in all 5 types of spices (Fig. 1). Black pepper has the maximum aflatoxigenic fungi contamination with *A. flavus* followed by *A. parasiticus. A. niger, A. ochraceus and P. verrucosum* (ochratoxigenic fungi) were also isolated from black pepper samples. Bokhari [18] has also isolated some of the similar fungi from black pepper and green cardamom samples from Saudi Arabia. Rizzo [19] has also reported that *Aspergillus flavus* and *A. parasiticus* were the predominant species isolated from Argentinean medicinal herbs. In present investigation, turmeric

samples were contaminated with *A. flavus, A. parasiticus, A. ochraceus* and *P. verrucosum* but *A. niger* was not isolated. It may be possible that the essential oil of turmeric inhibits the growth of *A. niger*. Few early reports show that turmeric has antimicrobial properties and it inhibit the growth of fungi [20]. Green cardamom, fennel and mace were also contaminated with *A. parasiticus, A. flavus, A. niger, A. ochraceus* and *P. verrucosum.*

Table 1: Percent incidence of isolated fungi from different spices

Name of fungi	Black pepper	Turmeric	Green cardamom	Fennel	Mace
Aspergillus parasiticus	5.9	3.1	1.8	3.4	2.5
A. oryzae	7.1	-	-	-	-
A. niger	19.5		3.7	5.8	12.5
A. flavus	25.3	14.3	9.5	7.3	10.7
A. ochraceus	10.1	5.4	2.4	4.7	-
A. versicolor	5.4	-	-	-	1.4
A. terreus	3.3	-	-	1.1	-
Penicillium citrinum	16.0	13.8	3.6	1.9	3.8
P. verrucosum	9.6	6.5	3.8	-	4.4
P. purpurogenum	1.6	-	-	-	-
Fusarium oxysporum	6.0	6.5	1.7	-	4.2
F. moniliforme	8.8	4.1	3.2	2.2	2.4
Rhizopus nigricans	5.0	-	-	-	6.3
R. oryzae	5.1	5.9	1.7	2.4	1.8
Mucor hiemalis	3.6	4.8	3.6	1.2	3.3

3.2 Aflatoxins and ochratoxin A producing potentiality of toxigenic fungi

Toxigenic fungi from different spices were examined for their toxicity and potentiality to produce aflatoxins and ochratoxin A (Table 2). It has been observed that, 52 % of *A. flavus* from black pepper was toxic and highly potential up to 22.9 µg/l (Fig. 2) followed by fennel (45.4%) and green cardamom (45%). All 5 types of spices have toxigenic *A. flavus* whereas toxigenic *A. parasiticus* were present only in black pepper, turmeric and fennel and none of the isolates from green cardamom were toxic. Our finding is well agreement with some other researchers [21]. In ochratoxin producing fungi, 40% isolates of *A. ochraceus* from black pepper and green cardamom were toxigenic with potentiality up to 12.0µg/l and 9.8µg/l. *A. niger* and *P. verrucosum* also produce ochratoxin in spice samples with potentiality up to 12.8µg/l and 13.8µg/l. Ochratoxin producing toxigenic strains of *A. niger, A. ochraceus* and *P. Verrucosum* were isolated from all the samples, in which they are present. Amézqueta [22] also reported similar finding and detected ochratoxin A producing fungi from foodstuffs.

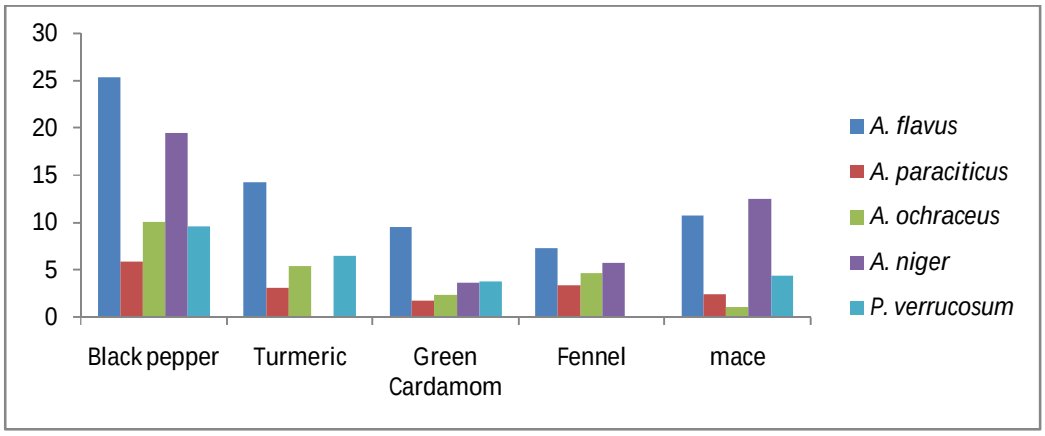

Figure 1: Percent incidence of ochratoxin A and aflatoxin producing fungi showing maximum contamination by *A. flavus* in black pepper.

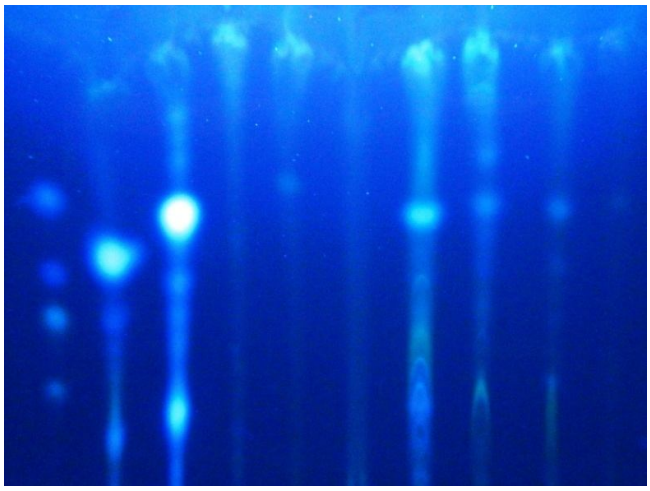

Figure 2: Spots of different aflatoxins on TLC plates from the sample of turmeric.

3.3 Natural occurrence of ochratoxin A and aflatoxins in spices

Ochratoxin A and aflatoxins, both were present in all types of spice samples. Table 3 shows that, 78.1% of the black pepper samples were contaminated with total aflatoxins with average amount of 320 ppb and only 56.3% were ochratoxin A contaminated. Hammami [23] has also reported that the black pepper from Qatar has also aflatoxins contamination and the detected level was cross the maximum level. In all the samples aflatoxin contamination is higher than ochratoxin A except in turmeric samples. In turmeric samples, 50.0% were ochratoxin A contaminated with average amount of 162 ppb and only 33.3% were contaminated with aflatoxins. It may be possible that essential oil of turmeric (ar-turmerone, α-turmerone and β-turmerone) inhibit the growth of *A. flavus* and aflatoxins production. Lowest amount of ochratoxin A was recorded in fennel samples, 10 ppb and only 22.8% were contaminated. Mace sample were also contaminated with both aflatoxins and ochratoxin A.

Table 2: Potentiality of aflatoxins and ochratoxin A producing fungi isolated from spices.

Fungal Species	Mycotoxin produced	Spices samples	No. of isolate analyzed	No. of isolate Positive / Percent toxicity	Potential Range(µg/l)
Aspergillus flavus	Aflatoxins	Black Pepper	25	13/52.0	0.8 – 22.9
		Turmeric	24	8/33.3	0.8 – 15.7
		Green Cardamom	20	9/45.0	3.4 – 16.8
		Fennel	22	10/45.4	1.2 – 6.3
		Mace	25	10/40.0	2.4 – 14.5
Aspergillus paraciticus	Aflatoxins	Black Pepper	25	11/44.0	2.1 – 5.6
		Turmeric	15	6/40.0	1.0 – 2.4
		Green Cardamom	10	0/0	-
		Fennel	10	3/30.0	2.1 – 10.5
		Mace	NF	--	-
Aspergillus ochraceus	Ochratoxin A	Black Pepper	15	6/40.0	1.4 – 12.0
		Turmeric	15	5/33.3	3.4 – 5.9
		Green Cardamom	15	6/40.0	2.4 – 9.8
		Fennel	15	4/26.6	3.0 – 7.4
		Mace	NF	--	-
Aspergillus niger	Ochratoxin A	Black Pepper	25	6/24.0	1.4 – 8.6
		Turmeric	NF	--	-
		Green Cardamom	15	3/20	1.5 – 6.9
		Fennel	25	6/24	2.4 – 9.5
		Mace	25	8/32	3.2 – 12.8
Penicillium verrucosum	Ochratoxin A	Black Pepper	20	6/30	2.4 – 6.5
		Turmeric	15	4/26.6	2.8 – 8.5
		Green Cardamom	15	5/33.3	3.0 – 13.8
		Fennel	NF	--	-
		Mace	15	4/26.6	2.4 – 8.0

Table 3: Natural occurrence of ochratoxin A and aflatoxins in spices.

Spices	No. of Samples analyzed	Aflatoxin		Ochratoxin A	
		N.P.S(% Cont)	Avg. amount (ppb)	N.P.S(% Cont)	Avg. amount (ppb)
B. Pep	55	43 (78.1)	320	31(56.3)	155
Tur	42	14(33.3)	134	21(50.0)	162
G. Card	32	23(71.8)	158	11(34.3)	68
Fenn	35	20(57.1)	95	08(22.8)	10
Mace	30	19(63.3)	185	18(60)	128

N.P.S - number of positive samples, % Cont – Percent contamination

3.4 Risk assessment of ochratoxin A and aflatoxins contamination on human health.

Aflatoxins are carcinogenic in nature and there are many reports regarding carcinogenicity in aflatoxins. Disease cause by aflatoxins causes aflatoxicosis they are mainly concern to livercerosis and other organ disorder sometime which are fatal. Ochratoxin is also a nephrotoxic in nature and its target organ is kidney. Over dose of ochratoxin can cause kidney failure and death also. EU has set the maximum limit of total aflatoxins up to 10 ppb and 15 ppb for ochratoxin in spices. But in our finding, all the contaminated spices samples are extremely contaminated with aflatoxins as well as ochratoxin A except fennel (Fig. 3). In fennel ochratoxin A contamination is less than the permissible limit. Hence, it can be considered safe for use. Both of the mycotoxins are highly toxic and fatal, when ingested by human beings or patients. So, it is important to monitor the spices before use.

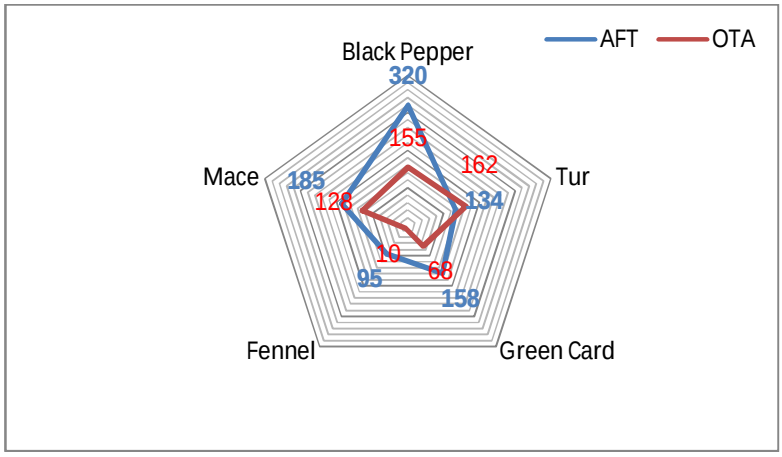

Figure 3: Amount of aflatoxins (AFT) and ochratoxin A (OTA) in spices.

IV. Conclusion

On the basis of the present study, it may be concluded that the spices are suitable substrate for fungal growth and further mycotoxin productions. All 5 types of spices were contaminated with aflatoxins and ochratoxin A. Black pepper is the most common spice used globally as spices had highest toxin concentration. Fennel has less contamination and the amount of ochratoxin A was under permissible limit hence, it is safe for consumption. Aflatoxins and ochratoxin A present in these spices were in sufficiently high concentration to induce carcinogenesis. 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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References

[1]. N Magan, A Medina and D Aldred. Possible Climate-change effects on mycotoxin contamination of food crops pre- and postharvest. Plant Pathology, 60(1), 2011, 150-163.
[2]. JW Bennett and M Klich. Mycotoxins. ClinIcal Microbiological Reviews, 16(3), 2003, 497–516.
[3]. Y Liu and F Wu. Global Burden of Aflatoxin-Induced Hepatocellular Carcinoma: A Risk Assessment. Environmental Health Perspectives, 118(6), 2010, 818-824.

[4]. P Jeswal. Ochratoxin A induced hepatorenal carcinogenesis in Mice (Mus musculus). Indian National Science Academy – B, 62, 1996, 339-344.
[5]. M Castegnaro, D Canadas, T Vrabcheva et al. Balkan endemic nephropathy: role of ochratoxion A through biomarkers. Molecular Nutrition & Food Research, 50(6), 2006, 519-529.
[6]. B Fazekas, A Tar and M Kovács. Aflatoxin and ochratoxin A content of spices in Hungary. Food Additives & Contaminants, 22(9), 2005, 856-863.
[7]. AK Roy, KK Sinha and HK Chourasia. Aflatoxin contamination of some common drug plants Applied and Environmental Microbiology, 54(3), 1988, 842-843.
[8]. AK Maren. Identification of common Aspergillus species.illustrated. CentraalbureauvoorSchimmelcultures, 2002.
[9]. JI Pitt. A laboratory guide to common Penicillium species. (2nd ed.). North Ryde: International Government Publication, 1988.
[10]. PE Nelson, TA Toussoun and WFO Marasas. Fusarium Species: an illustrated manual for identification. United State: Pennsylvania State University Press, 1983.
[11]. S Funder. Practical Mycology. Manual for identification of Fungi. (3rd ed.). United State: Haffner, 1958.
[12]. MA Pfaller, L Burmeister, MS Bartlett et al. Multicenter evaluation of four methods of yeast inoculums preparation. Journal of Clinical Microbiology, 26(8), 1988, 1437-1441.
[13]. UL Diener and ND Davis. Aflatoxin production by isolates Aspergillus flavus. Phytopathology, 56, 1966, 1390-1393.
[14]. ND Davis, GA Sansing, TV Ellenburg et al. Medium- Scale production and purification of ochratoxin A, a metabolite of Aspergillus ochraceus. Applied Microbiology, 23, 1972, 433-435.
[15]. TV Reddy, L Viswanathan and TA Venkitasubramanian. Thin layer chromatography of aflatoxins. Analytical Biochemistr,. 38, 1970, 568-571.
[16]. PM Scott, JW Lawrence and W Vanwalbeek. Detection of mycotoxin by thin layer chromatography: application to screening of fungal extract Applied Microbiology, 19, 1970, 839-842.
[17]. H Colak, EB Bingol, H Hampikyan et al. Determination of Aflatoxin Contamination in Red- Scaled, Red and Black Pepper by ELISA and HPLC. Journal of Food and Drug Analysis, 14(3), 2006, 92-296.
[18]. FM Bokhari. Spices Mycobiota and Mycotoxins Available in Saudi Arabia and Their Abilities to Inhibit Growth of Some Toxigenic Fungi. Mycobiology, 5(2), 2007, 47-53.
[19]. I Rizzo. Assessment of toxigenic fungi on Argentinean medicinal herbs. Microbiological Research, 159(2), 2004, 113-120.
[20]. FD Ferreira, SAG Mossini, FMD Ferreira. The inhibitory Effects of Curcuma longa L. Essential Oil and Curcumin on Aspergillus flavus Link Growth and Morphology. The Scientific World Journal, 2013, 6pages.
[21]. M Hashem and S Alamri. Contamination of common Saudi Arabia market with potential mycotoxin-producing fungi. Saudi Journal of biological Science, 17(2), 2010, 165-175.
[22]. S Amézqueta, S Schorr-Galindo, Murillo-ArbizuMn et al. OTA-producing fungi in foodstuffs: A review. Food Control, 26(2), 2012, 259-268.
[23]. W Hammami, S Fiori, RA Thani et al. Fungal and aflatoxin contamination of marketed spices. Food Control, 37, 2014, 177-181.