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Investigation of Aflatoxin B_1 in Spices Marketed in Hyderabad, India by ELISA Method

Amir Sasan Mozaffari Nejad[1], Mansour Bayat[2] and Ali Asghar Ahmadi[3]*

[1]Young Researchers and Elite Club, Sari Branch, Islamic Azad University, Sari, Iran.
[2]Department of Medical and Veterinary Mycology,Faculty of Veterinary Specialized Sciences, Islamic Azad University, Science and Research Branch, Tehran, Iran.
[3]North Research Center-Pasteur Institute of Iran, Amol, Iran.

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Eighteen samples of spices (6 whole chilli, 6 whole black pepper, and 6 black pepper powder) purchased from three popular markets of Hyderabad in India were analysed for presence of aflatoxin B_1. Enzyme-linked immunosorbent assay (ELISA) method was used for the purpose of analysing the samples. All the analyses were done twice. Aflatoxin B_1 was found in all of the samples and the concentration of aflatoxin ranged from 31.15 to 174.68 ng/kg. The mean AFB_1 concentration in black pepper powder was significantly higher ($P < 0.05$) than that in whole chilli and black pepper powder. There was no significant difference between the mean AFB_1 concentrations in whole chilli and black pepper powder. However, none of the samples exceeded the maximum limit of 5000ng/kg prescribed in European Union regulations for aflatoxin B_1. There is considerable scope for betterment in spices production in India.

Key words: Aflatoxin B_1, Spices, ELISA, India.

During the recent decades, measures have been taken to overcome the problems attributed to chemical side-effects. Thus, people again turned to natural products especially in pharmaceutical and food industry (Mozaffari Nejad *et al.*, 2013). Traditionally, spices and herbs are valued for their distinctive flavors, colors and aromas and are the most versatile substances widely used all over the world (Hashem and Alamri, 2010). Spices are considered as the important crops in India and its Guntur region in Andhra Pradesh is the largest chilli producing area (Ravi Kiran *et al.*, 2005). They are commonly used in Indian culinary practices and many of them have antioxidant and antimicrobial effects (Sharma *et al.*,2012). India is the major exporter of the chillies, and China, Spain, Mexico, Pakistan and Turkey stand in the next places, respectively (Iqbal *et al.*, 2010). Mycotoxins contamination of spices is a serious hazard throughout the world that can affect international trade of spices. Fungal deterioration of stored seeds and grains is a chronic problem in the Indian storage system because of India's tropical hot and humid climate (Reddy *et al.*,2009a).Aflatoxins are the most important mycotoxins, recognized as ubiquitous contaminants of food throughout the developing world (Kamkar *et al.*, 2013).The major aflatoxins are AFB_1, AFB_2, AFG_1, AFG_2 and two more additional metabolic products, M_1 and M_2(Samuel *et al.*,2013).Among them, aflatoxin B_1 (AFB_1) is the most potent case of human carcinogen; hence, the International Agency for Research on Cancer (IARC) classified AFB_1 into a primary group of carcinogenic compounds (Reddy *et al.*, 2009a; Tavakoli *et al.*,2013).

At least 100 countries have regulations to control major mycotoxins, especially aflatoxins,

* To whom all correspondence should be addressed.
Mob.: +98912 422 2576;
E-mail: ahmadi.pasteur@gmail.com

in commodities and food, so that the maximum tolerable mycotoxins levels vary greatly among the countries (Reddy *et al.*, 2009b). European Union has established the maximum tolerable limits for AFs in spices as 10 µg/kg for total aflatoxins (B_1 + B_2 + G_1 + G_2) and 5 µg/kg for AFB_1(Commission Regulation, 2002).

By now, several methods such as thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC) and enzyme-linked immunosorbent assay (ELISA) have been introduced for determination of aflatoxin (Reddy *et al.*, 2009b; Tavakoli *et al.*, 2012). ELISA method was recently presented and used mainly for routine analyses. ELISA is a simple method which enjoys some advantages as portability of the equipment, hand-holding validation, and reliability for the analysis of a large number of samples (Guan *et al.*, 2011).

Several studies yet have been conducted on natural occurrence of aflatoxins and AFB_1 in spices in India, Iran, Pakistan, and Turkey. Hence, this study aimed to explore contamination of spices with aflatoxin B_1 in Hyderabad region, India using ELISA method.

MATERIALS AND METHODS

Samples

In June 2012, a total of 18 samples of commercially available spices were randomly purchased from three popular markets in Hyderabad, Andhra Pradesh Province in India. The samples included whole black pepper (N=6), black pepper powder (N=6) and whole chilli powder (N=6). 100 g of each samples werestored at 4-6 °C in plastic bags until the analysis.

Analysis of AFB_1 by ELISA

The quantitative analysis of AFB_1 in the samples was performed based on a competitive enzyme immunoassay by using RIDASCREEN® Aflatoxin B_1 30/15 (Art. No: R1211, R-Biopharm, Darmstadt, Germany) test kit. Preparation of the samples and ELISA test were performed according to the method described by R-Biopharm GmbH (2010).

Sample preparation

Sample preparation and separation with aflatoxin column were preformed according to the instructions of the test kit manual (RidascreenAflatoxin B_1 30/15) (R-Biopharm GmbH, 2010). 25 ml of methanol (70%) was added to 5 g of spices. Afterwards the samples were vigorously shaked for three minutes manually. The achieved extract was filtered through a filter paper and diluted with distilled water (1:1). At last, 50 µl of the diluted filtrate per well was used in the test.

ELISA Test procedure

According to RidascreenAflatoxin B_1 30/15 (Art No.: 1211) test kit manual, 50 µl of the standard solution or prepared sample in *duplicate* was added to the wells of micro-titer plate. Then 50 µl of the enzyme conjugate and 50 µl of the anti-aflatoxin antibody solution were added to each well, mixed gently and incubated for 30 min at room temperature (20-25 °C). Liquid was removed from wells by tapping the wells upside down vigorously against the absorbent paper; the wells were then washed by a washing buffer (250 µl) twice. After the washing step, 100 µl of substarte/chromogen solution was added to each well, mixed gently and incubated for 15 min at room temperature (20-25°C) in a dark place. Finally, 100 µl of the stop solution (1N H_2SO_4) was added to each well and the absorbance was measured at 450 nm in ELISA plate reader.

Statistical analysis

The data were analysed by Statistics 16 SPSS IBM software. Moreover, to evaluate the differences in mean values, three groups of samples were used for *t-test* and ANOVA was used for independent samples. The differences among the mean values were found to be significant at $P< 0.05$.

RESULTS AND DISCUSSION

The occurrence and levels of AFB_1 in spice samples consisting of whole chilli powder, black powder and whole black samples collected from India are presented in Table 1. Aflatoxin B_1 was found in all spices sample, ranging from 31.15 to 174.68 ng/kg. Also, none of the samples exceeded the European Union limit of 5000 ng/kg for aflatoxin B_1. The mean AFB_1 concentration in black powder was significantly higher ($P < 0.05$) than whole chilli and black pepper powder samples. However, no significant difference was observed in the mean AFB_1 concentration in the whole chilli and black pepper powder.

In overall, spices are mainly produced and consumed in developing and developed countries (Paterson, 2007).As can be seen in Table 2, many cases of aflatoxin B_1 contamination in spices have been reported in the studies conducted by other researchers (Reddy *et al.,* 2001; Omurtag *et al.,* 2002; Fazekas *et al.,*2005; Colak *et al.,* 2006; Aydin e*t al.,* 2007; Cho *et al.,* 2008; Iqbal *et al.,* 2010; Iqbal *et al.,* 2011; Jalili and Jinap, 2012; Golge *et al.,* 2013). Among aflatoxins, AFB_1 is the most frequent toxin in spices with higher levels and chilli samples are the most frequent contaminated substrate (Ardic *et al.,*2008).In a previous study, Saha *et al.* (2007) from India reported that by ELISA method 2 (13%) out of total 16 samples of chillies contaminated with aflatoxin B_1 ranged from 1.8-8.4 µg/kg, but our results were found to be higher than these results. In a similar study in Pakistan by Paterson (2007) has analysed 9 chilli samples and 9 (100%) samples were found contaminated of aflatoxin B_1 with mean level of 6.8-96.2 µg/kg, which is similar with our results.

In comparison, several studies have been reported on the contamination of spices with aflatoxin B_1.

According to, In Spain, Hernández Hierro *et al.*(2008) found aflatoxin B_1 in 90% of red paprika with average concentration of 1.1 µg/kg. In another study by Ozbey and Kabak (2012) analysed 22 red chilli samples and found that 63.6% of red chilli powder contained AFs at detectable levels and 3 red chilli powder exceeded the European Union regulatory limit for aflatoxin B_1. Salari *et al.* (2011) in Sabzevar (a city in Khorasan Razavi province in Iran), 36 samples of red pepper were considered and the incidence of AFB_1 and Ochratoxin A was respectively (28) 77.8% and (8) 22.2% within the range of 1.1-15.0 µg/kg and 0.59-2.35 µg/kg as it

Table 1. The occurrence of aflatoxin B_1 (AFB_1) in spices in India

Sample category	Number of samples: 18			
	Sample tested, *n*	Minimum(ng/kg)	Maximum(ng/kg)	Mean ± SE[a](ng/kg)
Black Pepper Powder	6	48.35	174.68	123.70[a] ± 18.20
Whole Chilli	6	33.55	41.49	38.86[a] ± 1.30
Whole Black Pepper	6	31.15	44.12	36.52[a] ± 2.31
Total	18	31.15	174.68	66.36 ± 7.27

[a]Mean ± SE(Standard Error) with different letters is significantly different

Table 2. Incidence and levels of AFB_1 in spices samples in different countries.

Reference	Country	Products	No. of Samples	Positive n (%)	Method	Myco-toxin	Range (µg/kg)
Reddy *et al*. (2001)	India	Chilli	182	107 (59)	ELISA	AFB_1	<10-969
Omurtag *et al*. (2002)	Turkey	Red pepper	26	17 (65)	HPLC-FD	AFB_1	0.6-56
Fazekas *et al*. (2005)	Hungary	Ground red pepper	70	18 (26)	HPLC-FD	AFB_1	0.14-15.7
Colak *et al*. (2006)	Turkey	Red pepper	30	6 (20)	ELISA	AFB_1	2.9-11.2
		Black Pepper	24	2 (8.4)			9.8-10.3
Aydin *et al*. (2007)	Turkey	Powdered red pepper	100	68 (68)	ELISA	AFB_1	0.025-40.9
Cho *et al.* (2008)	Korea	Black Pepper	2	0	HPLC-FD	AFB_1	ND
		Red pepper	41	7 (17)			0.08-4.45
Iqbal *et al*. (2010)	Pakistan	Whole chilli	22	16 (73)	HPLC-FD	AFB_1	<0.05-96.3
		Chilli Powder	22	19 (86)			<0.05-89.6
Jalili and Jinap (2012)	Malaysia	Dried chilli	80	52 (65)	HPLC-FD	AFB_1	0.2-56.61
Golge *et al*. (2013)	Turkey	Chilli	182	150 (82.4)	HPLC-FD	AFB_1	0.24-165

was determined by ELISA. In comparison, HPLC detected AFB_1 and OTA in 25 (69.4%) and 6 (16.7%) of samples within the range of 0.4-14.5 µg/kg and 0.74-2.17 µg/kg, respectively. According to Ardic *et al.* (2008), in Turkey, aflatoxin B_1 in 72 (96%) out of total 75red ground pepper samples was in the range of 0.11 and 24.7 µg kg^{-1}. Shundo *et al.* (2009) from Brazil reported that 82.9% of paprika samples were AFs positive, and AFB_1 was detected in 61.4% of the samples at the levels ranging from 0.5 to 7.3 µg/kg with the mean concentration of 3.4 µg/kg. In previous studies from Pakistan, Iqbal *et al*.(2013) observed that 85 (50%) out of total 170 samples of chillies were contaminated with aflatoxins. Furthermore, in Morocco, 14 red paprika pepper samples were screened for aflatoxin contamination and 14 (100%) of the samples contained aflatoxin B_1. The aflatoxin of spice was in the range of 2.88 and 5.40 µg/kg (Zinedine *et al.*, 2006).

CONCLUSION

In the present study, high-risk levels of aflatoxin B_1, which is a serious threat to human health, in the spices in India were investigated. It was concluded that the growing conditions, harvesting, processing methods, storage conditions and post-harvest treatments should be closely controlled by the public health authorities in India to prevent aflatoxin contamination risks posed to spices. Also, it would be helpful to address questions such as whether the current regulatory standards applied to aflatoxin levels in diets protect human health and to what extent aflatoxin exposure contributes to hepatocellular carcinoma.

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REFERENCES

1. Ardic, M., Karakaya, Y., Atasever, M., Durmaz, H., Determination of aflatoxinB_1levels in deep-red ground pepper (isot) using immunoaffinity column combined with ELISA. *Food and Chem Toxicol*, 2008; **46**: 1596-1599.
2. Aydin, A., Erkan, M., Baskaya, R., Ciftcioglu, G., Determination of aflatoxin B_1 levels in powdered red pepper. *Food Control*, 2007; **18**: 1015-1018.
3. Cho, S.-H., Lee, C.-H., Jang, M.-R., Son, Y.-W., Lee, S.-M., Choi, I.-S., et al.,Aflatoxins contamination in spices and processed spice products commercialized in Korea. *Food Chem*, 2008; **107**: 1283-1288.
4. Colak, H., Bingol, E. B., Hampikyan, H., Nazli, B., Determination of aflatoxin contamination in red-scaled, red and black pepper by ELISA and HPLC. *J Food Drug Anal*, 2006; **14**: 292-296.
5. Commission Regulation (EC) No 472/2002. *Official J Europ Comm*, **75**: 18-20.
6. Fazekas, B., Tar, A., Kovacs, M.,Aflatoxin and ochratoxin A content of spices in Hungary. *Food Add Contamin*,2005; **22**: 856-863.
7. Golge, O., Hepsag, F.,Kabak, B., Incidence and level of aflatoxin contamination in chilli commercialised in Turkey. *Food Control*, 2013; **33**: 514-520.
8. Guan, D., Li, P., Zhang, Q., Zhang, W., Zhang, D., Jiang, J., An ultra-sensitive monoclonal antiboday-based competitive enzyme immunoassay for aflatoxin M_1 in milk and infant milk products. *Food Chem*, 2011; **125**: 1359-1364.
9. Hashem, M., Alamri, S., Contamination of common spices in Saudi Arabia markets with potential mycotoxin-producing fungi. *Saudi J Biolog Sci*, 2010; **17**: 167-175.
10. Hernández Hierro, J.M., Garcia-Villanova, R.J., Rodríguez Torrero, P., Toruño Fonseca, I.M.,Aflatoxins and ochratoxinA in red paprika for retail sale in Spain: occurrence and evaluation of a simultaneous analytical method. *J Agri Food Chem*, 2008; **56**: 751-756.
11. Iqbal, S.Z., Paterson, R.R.M., Bhatti, I.A., Asi, M.R., Sheikh, M.A., Bhatti, H.N., Aflatoxin B_1 in chillies from the Punjab region, Pakistan. *Mycotox Res*, 2010; **26**: 205-209.
12. Iqbal, S.Z., Asi, M.R., Zuber, M., Akhtar, J., Saif, M.J., Natural occurrence of aflatoxins and ochratoxin A in commercial chilli and chilli sauce samples. *Food Control*, 2013; **30**: 621-625.
13. Jalili, M., Jinap, S., Natural occurrence of aflatoxins and ochratoxinA in commercial dried chili. *Food Control*, 2012; **24**: 160-164.
14. Kamkar, A., Yazdankhah, S., Mohammadi Nafchi, A., MozaffariNejad, A.S.,Aflatoxin M_1 in raw cow and buffalo milk in Shush city of Iran. *Food Add Contamin: Part B*, 2013;http://dx.doi.org/10.1080/19393210.2013.830277.
15. Mozaffari Nejad, A.S., Kamakr, A., Giri, A., Pourmahmoudi, A.A., Ethnobotany and folk medicinal uses of major trees and shrubs in Northern Iran. *J Med Plants Res*, 2010; **7**: 284-

289.

16. Omurtag, G.Z., Atak, G., Keskin, G., Ersoy, O.,HPLC assay for aflatoxins in dried red peppers and feedstuffs in Turkey. *Acta Pharmaceutica Turcica,* 2002; **44**: 11-22.
17. Ozbey, F.,Kabak, B., Natural co-occurrence of aflatoxins and ochratoxin A in spices.*Food Control,* 2012; **28**: 354-361.
18. Paterson, R.R.M.Aflatoxins contamination in chili samples from Pakistan. *Food Control,* 2007; **18**: 817-820.
19. Ravi Kiran, D., Narayana, K.J.P., Vijayalakshmi, M.,Aflatoxin B_1 production in chillies (*Capsicum annuum* L.) kept in cold stores. *Afr J Biotechnol,* 2005; **4**: 791-795.
20. R-BiopharmGmbH .Enzyme immunoassay for the quantitative analysis of aflatoxin B_1 Art. No.: R1211. Darmstadt, Germany, 2010.
21. Reddy, K.R.N., Reddy, C.S., Muralidharan, K., Potential of botanicals and biocontrol agents on growth and aflatoxin production by *Aspergillus flavus* infecting rice grains. *Food Control,* 2009a; **20**: 173-178.
22. Reddy, K.R.N., Reddy, C.S., Muralidharan, K., Detection of Aspergillus spp.And aflatoxin B1 in rice in India. *Food Microbiology,*2009b; **26**: 27-31.
23. Reddy, S.V., Mayi, D.K., Reddy, M.U., Thirumala-Devi, K., Reddy, D.V.R.,Aflatoxins B_1 in different grades of chillies (Capsicum annum L.) in India as determined by indirect competitive ELISA. *Food Add Contamin,* 2001; **18**: 553-558.
24. Saha, D., Acharya, D., Roy, D., Shrestha, D., Dhar, T. K., Simultaneous enzyme immunoassay for the screening of aflatoxin B_1 and ochratoxin A in chili samples. *Analytica Chimica Acta,* 2007; **584**: 343-349.
25. Salari, R., HabibiNajafi, M.B., Boroushaki, M.T., Mortazavi, S.A., FathiNajafi, M., A comparison between ELISA and HPLC for aflatoxin B_1 and ochratoxin A detection in Iranian red pepper. *J Food Res,* 2012; **21**: 481-491.
26. Samuel, S.M., Aiko, V., Panda, P., Mehta, A.,Aflatoxin B_1 occurrence, biosynthesis and its degradation. *J Pure Appl Microbio,* 2013; **7**: 965-971.
27. Sharma, J., Pazhaniandi, P.P., Tanwar, V.K., Das, S.K., Goswami, M., Antioxidant effect of turmeric powder, nitrate and ascorbic acid on stored chicken mince. *Inter J Food Sci Technol,* 2012; **47**: 61-66.
28. Shundo, L., Almeida, A.P.D., Alaburda, J., Lamardo, L.C.A., Navas, S.A., Ruvieri, V., et al.,Aflatoxins and ochratoxin A in Brazilian paprika. *Food Control,* 2009; **20**: 1099-1102.
29. Tavakoli, H.R., Riazipour, M., Kamkar, A., Rafati, H., MozaffariNejad, A.S., Occurrence of aflatoxin M_1 in cheese samples from Tehran, Iran. *Food Control*, 2012; **23**: 293-295.
30. Tavakoli, H. R., Kamkar, A., Riazipour, M., MozaffariNejad, A. S.,Rafati, H., Assessment of aflatoxin M_1 levels by Enzyme-linked Immunosorbent Assay in yoghurt consumed in Tehran, Iran. *Asian J Chem*, 2013; **25**: 2836-2838.
31. Zinedine, A., Brera, C., Elakhdari, S., Catano, C., Debegnach, F., Angelini, S., et al., Natural occurrence of mycotoxins in cereals and spices commercialised in Morocco. *Food Control,* 2006; **17**: 868-874.