

## Investigation of Aflatoxin B<sub>1</sub> in Spices Marketed in Hyderabad, India by ELISA Method

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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<sub>1</sub>. Enzyme-linked immunosorbent assay (ELISA) method was used for the purpose of analysing the samples. All the analyses were done twice. Aflatoxin B<sub>1</sub> was found in all of the samples and the concentration of aflatoxin ranged from 31.15 to 174.68 ng/kg. The mean AFB<sub>1</sub> 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<sub>1</sub> 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<sub>1</sub>. There is considerable scope for betterment in spices production in India.**

**Key words:** Aflatoxin B<sub>1</sub>, 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<sub>1</sub>, AFB<sub>2</sub>, AFG<sub>1</sub>, AFG<sub>2</sub> and two more additional metabolic products, M<sub>1</sub> and M<sub>2</sub> (Samuel *et al.*, 2013). Among them, aflatoxin B<sub>1</sub> (AFB<sub>1</sub>) is the most potent case of human carcinogen; hence, the International Agency for Research on Cancer (IARC) classified AFB<sub>1</sub> 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,

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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<sub>1</sub> + B<sub>2</sub> + G<sub>1</sub> + G<sub>2</sub>) and 5 µg/kg for AFB<sub>1</sub> (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<sub>1</sub> in spices in India, Iran, Pakistan, and Turkey. Hence, this study aimed to explore contamination of spices with aflatoxin B<sub>1</sub> 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 were stored at 4-6 °C in plastic bags until the analysis.

### Analysis of AFB<sub>1</sub> by ELISA

The quantitative analysis of AFB<sub>1</sub> in the samples was performed based on a competitive enzyme immunoassay by using RIDASCREEN® Aflatoxin B<sub>1</sub> 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 performed according to the instructions of the test kit manual

(RidascreenAflatoxin B<sub>1</sub> 30/15) (R-Biopharm GmbH, 2010). 25 ml of methanol (70%) was added to 5 g of spices. Afterwards the samples were vigorously shaken 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<sub>1</sub> 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 substrate/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<sub>2</sub>SO<sub>4</sub>) 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<sub>1</sub> in spice samples consisting of whole chilli powder, black powder and whole black samples collected from India are presented in Table 1. Aflatoxin B<sub>1</sub> 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<sub>1</sub>. The mean AFB<sub>1</sub> 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<sub>1</sub> 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<sub>1</sub> 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 *et 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<sub>1</sub> 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<sub>1</sub> 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<sub>1</sub> 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<sub>1</sub>.

According to, In Spain, Hernández Hierro *et al.* (2008) found aflatoxin B<sub>1</sub> 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<sub>1</sub>. 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<sub>1</sub> 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<sub>1</sub> (AFB<sub>1</sub>) in spices in India

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

<sup>a</sup>Mean ± SE (Standard Error) with different letters is significantly different

**Table 2.** Incidence and levels of AFB<sub>1</sub> in spices samples in different countries.

Reference	Country	Products	No. of Samples	Positive n (%)	Method	Myco-toxin	Range (µg/kg)
Reddy <i>et al.</i> (2001)	India	Chilli	182	107 (59)	ELISA	AFB <sub>1</sub>	<10-969
Omurtag <i>et al.</i> (2002)	Turkey	Red pepper	26	17 (65)	HPLC-FD	AFB <sub>1</sub>	0.6-56
Fazekas <i>et al.</i> (2005)	Hungary	Ground red pepper	70	18 (26)	HPLC-FD	AFB <sub>1</sub>	0.14-15.7
Colak <i>et al.</i> (2006)	Turkey	Red pepper	30	6 (20)	ELISA	AFB <sub>1</sub>	2.9-11.2
		Black Pepper	24	2 (8.4)			9.8-10.3
Aydin <i>et al.</i> (2007)	Turkey	Powdered red pepper	100	68 (68)	ELISA	AFB <sub>1</sub>	0.025-40.9
Cho <i>et al.</i> (2008)	Korea	Black Pepper	2	0	HPLC-FD	AFB <sub>1</sub>	ND
		Red pepper	41	7 (17)			0.08-4.45
Iqbal <i>et al.</i> (2010)	Pakistan	Whole chilli	22	16 (73)	HPLC-FD	AFB <sub>1</sub>	<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 <sub>1</sub>	0.2-56.61
Golge <i>et al.</i> (2013)	Turkey	Chilli	182	150 (82.4)	HPLC-FD	AFB <sub>1</sub>	0.24-165

was determined by ELISA. In comparison, HPLC detected AFB<sub>1</sub> 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<sub>1</sub> in 72 (96%) out of total 75 red ground pepper samples was in the range of 0.11 and 24.7 µg kg<sup>-1</sup>. Shundo *et al.* (2009) from Brazil reported that 82.9% of paprika samples were AFs positive, and AFB<sub>1</sub> 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<sub>1</sub>. 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<sub>1</sub>, 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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