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 AFB₁, AFB₂, AFG₁, AFG₂, and two more additional metabolic products, M₁ and M₂ (Samuel et al., 2013). Among them, aflatoxin B₁ (AFB₁) is the most potent case of human carcinogen; hence, the International Agency for Research on Cancer (IARC) classified AFB₁, 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,
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 were stored 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, 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 (RidascreenAflatoxinB, 30/15)(R-Biopharm GmbH, 2010). 25 ml of methanol \( (70\%) \) was added to 5 g of spices. Afterwards the samples were vigorously shook 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, 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 \( (20-25 \, ^oC) \). 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 \( (250 \, \mu l) \) 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 \( (20-25^oC) \) 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, 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 B₁ 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, AFB₁ 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 B₁ 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 B₁ 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 B₁. According to, In Spain, Hernández Hierro et al. (2008) found aflatoxin B₁ 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 B₁. 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 AFB₁ 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 B₁ (AFB₁) in spices in India

<table>
<thead>
<tr>
<th>Sample category</th>
<th>Number of samples: 18</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample tested, n</td>
<td>Minimum(ng/kg)</td>
</tr>
<tr>
<td>Black Pepper Powder</td>
<td>6</td>
</tr>
<tr>
<td>Whole Chilli</td>
<td>6</td>
</tr>
<tr>
<td>Whole Black Pepper</td>
<td>6</td>
</tr>
<tr>
<td>Total</td>
<td>18</td>
</tr>
</tbody>
</table>

*Mean ± SE(Standard Error) with different letters is significantly different*

### Table 2. Incidence and levels of AFB₁ in spices samples in different countries.

<table>
<thead>
<tr>
<th>Reference</th>
<th>Country</th>
<th>Products</th>
<th>No. of Samples</th>
<th>Positive n (%)</th>
<th>Method</th>
<th>Mycotoxin</th>
<th>Range (µg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reddy et al. (2001)</td>
<td>India</td>
<td>Chilli</td>
<td>182</td>
<td>107 (59)</td>
<td>ELISA</td>
<td>AFB₁</td>
<td>&lt;10-969</td>
</tr>
<tr>
<td>Omurtag et al. (2002)</td>
<td>Turkey</td>
<td>Red pepper</td>
<td>26</td>
<td>17 (65)</td>
<td>HPLC-FD</td>
<td>AFB₁</td>
<td>0.6-56</td>
</tr>
<tr>
<td>Fazekas et al. (2005)</td>
<td>Hungary</td>
<td>Ground red pepper</td>
<td>70</td>
<td>18 (26)</td>
<td>HPLC-FD</td>
<td>AFB₁</td>
<td>0.14-15.7</td>
</tr>
<tr>
<td>Colak et al. (2006)</td>
<td>Turkey</td>
<td>Red pepper</td>
<td>30</td>
<td>6 (20)</td>
<td>ELISA</td>
<td>AFB₁</td>
<td>2.9-11.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Black Pepper</td>
<td>24</td>
<td>2 (8.4)</td>
<td></td>
<td></td>
<td>9.8-10.3</td>
</tr>
<tr>
<td>Aydin et al. (2007)</td>
<td>Turkey</td>
<td>Powdered red pepper</td>
<td>100</td>
<td>68 (68)</td>
<td>ELISA</td>
<td>AFB₁</td>
<td>0.025-40.9</td>
</tr>
<tr>
<td>Cho et al. (2008)</td>
<td>Korea</td>
<td>Black Pepper</td>
<td>2</td>
<td>0</td>
<td>HPLC-FD</td>
<td>AFB₁</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Red pepper</td>
<td>41</td>
<td>7 (17)</td>
<td>HPLC-FD</td>
<td>AFB₁</td>
<td>0.08-4.45</td>
</tr>
<tr>
<td>Iqbal et al. (2010)</td>
<td>Pakistan</td>
<td>Whole chilli</td>
<td>22</td>
<td>16 (73)</td>
<td>HPLC-FD</td>
<td>AFB₁</td>
<td>&lt;0.05-96.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Chilli Powder</td>
<td>22</td>
<td>19 (86)</td>
<td>HPLC-FD</td>
<td>AFB₁</td>
<td>&lt;0.05-89.6</td>
</tr>
<tr>
<td>Jalili and Jinap (2012)</td>
<td>Malaysia</td>
<td>Dried chilli</td>
<td>80</td>
<td>52 (65)</td>
<td>HPLC-FD</td>
<td>AFB₁</td>
<td>0.2-56.61</td>
</tr>
<tr>
<td>Golge et al. (2013)</td>
<td>Turkey</td>
<td>Chilli</td>
<td>182</td>
<td>150 (82.4)</td>
<td>HPLC-FD</td>
<td>AFB₁</td>
<td>0.24-165</td>
</tr>
</tbody>
</table>
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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