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Incidence of Aflatoxin B_1, B_2, G_1, G_2 in Spices Marketed in Karachi, Pakistan

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Abstract

Aflatoxins form one of the vital groups of mycotoxins. Aflatoxins are one of the highly poisonous secondary metabolites of A. flavus *and* A. parasiticus. *In the current study aflatoxinaAFB1, AFB2, AFG1 and AFG2 contamination in the spices purchased from local market located at all five towns of Karachi, Pakistan; have been analyzed using standard method of analysis (HPLC) technique. A total of 360 samples of spices were tested for each aflatoxin. 57 of the spices samples were detected as positive for aflatoxin B_1, 52 samples were found to be contaminated with AF B_2, 35 samples of spices were being tested as positive with AFG_1. Whereas Aflatoxin G_2 was found to be absent in all samples. Maximum level of aflatoxin B_1 was detected as 8.3 – 20.62ppm in turmeric and minimum level was found in coriander that is 0.66 – 0.98ppm. Maximum level of aflatoxin B_2 was detected as 9.8 – 19.64ppmin turmeric and minimum level was found in coriander that is 0.48 – 1.0ppm. Maximum level of aflatoxin B_1 was detected as 1.22 – 6.46 in red chili and minimum level was found in black pepper that is 1.42 – 2.54ppm. Most of the samples (109 out of 360) in the currents study found to contain aflatoxin within the threshold limit set for aflatoxin B1. It can be concluded that the most of the spices marketed in Karachi are safe for consumption. The high levels of aflatoxin in spice samples detected in the period of studies could be a serious risk to human health in the largest populated city of Pakistan, where limited resources are available for the prevention and controlling their levels in the food supply.*

Keywords: Mycotoxin risk, Food safety, Aspergillus, Aflatoxin, Spices

Introduction

Aflatoxins, one of the vital groups of mycotoxins, are metabolites of fungus *A. flavus* and *A. parasiticus*. Aflatoxin is produced during harvesting, production, processing, and storage of various food commodities. Aflatoxins are reported to be the carcinogenic, immunosuppressive, immunotoxic, hepatotoxic, and teratogenic agent (Reddy, Kiran Mayi *et al.* 2001).

Black pepper *(P. nigrum)* is well known as a "King of Spices" and it is the most frequently used spice among all the spices (Ravindran and Kallupurackal 2012). In historical times, this spice is also reported to have digestive power, to boost appetite, and to cure cold, cough, diseases of the throat, intermittent fever, colic, dysentery, worms and pile (Kunnumakkara, Koca *et al.* 2009). It is also reported to possess antimicrobial properties. The black pepper contains antioxidants and piperine and, found to impose beneficial effects on nervous system. Cinnamon (*Cinnamomum verum*) is one of the finest sweet spices made from the inner bark of trees (Singletary 2010). Cinnamon is broadly utilized in Chinese herbal medicine and is claimed to offer a variety of therapeutic and calming effects. Cinnamon's characteristic aroma and flavour are derived from cinnamaldehyde, an essential oil found in the bark. Cinnamaldehyde has antibacterial, antiviral, and antifungal effects (Al-juraifani 2011). Cinnamon has excellent anti-fungal properties. Cinnamon hasbeen reported to reduces blood sugar levels, improve stability against insulin hormone and protect against HIV as well as it lower risk

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of heart diseases(Al-juraifani 2011). Nutmeg (*Myristica fragrans*) is a fragrant spice that has been regarded antiquity for its aromatic, aphrodisiac, and medicinal powers (Agbogidi and Azagbaekwe 2013). Nutmeg contains potent anti-inflammatory chemicals that also function as antioxidants, in addition to its various culinary purposes. Though further research on these effects in humans is needed, they may benefit mood, blood sugar control, and heart health (Sembiring 2020). Coriander (*Coriandrum sativum*) is an annual herb and a spice. Coriander seeds are reported to be carminative, diuretic, antibilious, refrigerant and aphrodisiac. Studies recommend that coriander controls blood sugar, cholesterol and free radical production (Sharma and Sharma 2012).

Garlic (*Allium sativum*) is possibly the maximum broadly quoted herb with medicinal potentials regarded in the literature. Garlic has been shown to display antifungal, antibacterial, hypoglycemic, hypolipidemic, and ant atherosclerotic properties (Agarwal 1996). Chili (*Capsicum Annum*) is a commercially important spice, having diverse range of forms and sizes, as well as sensory characteristics such as colour, pungency, and piquancy, which make otherwise bland mass nutritious flesh, cereal, and vegetable dishes more appealing. Chilies with a moderate pungency have been used in salads, pickles, cooking, and as food colourants, whereas chillies with a hotter pungency have been employed as food colourants (Gupta, Ahmed *et al.* 2002). Finely divided powdered capsicum and capsicum tincture solution, has been used to relieve postoperative nausea, sore throat, and vomiting (Sachan, Kumar *et al.* 2018). Turmeric (*Curcuma longa*) is another spice utilized in Asian cuisine, medicine, cosmetics, and textile and fabric colouring. Turmeric has been used for centuries as a traditional remedy to treat a variety of ailments, including rheumatism, body aches, skin diseases, intestinal worms, diarrhea, intermittent fevers, hepatic diseases, urinary discharges, dyspepsia, inflammations, constipation, leukoderma, amenorrhea, dental diseases, digestive problems like dyspepsia and acidity, indigestion, flatulence, ulcers, and colic inflammatory disorders such as arthritis, colitis and hepatitis (Sachan, Kumar *et al.* 2018).

In a study, 75 examples of various spices were analyzed for aflatoxin using two distinctive insightful techniques. 27 paprika, all the chili powder and four ground black pepper tests were debased with aflatoxin B1 in the scope of 0.5– 116.4, 1.6–80.4 and 0.3–1.2μgkg^{-1} separately. 23 (30%) paprika and chili powder tests were over as far as possible utilized in the European Union. No aflatoxin defilement was recognized in the cumin tests at a location cut off of 0.2μgkg^{-1} (Sachan, Kumar *et al.* 2018). In another study 27 fragrant herbs, 28 spices and 48 herbal implantations and prescription plants were investigated for assessment of aflatoxins by (HPLC) high performance liquid chromatography utilizing a post-section derivatisation method and a fluorescence location. Of the 103 examples investigated just 7 spices came about positives: 5 chili peppers, 1 cinnamon and 1 nutmeg (Romagnoli, Menna *et al.* 2007). In a study carried out to determine the aflatoxin contamination in spices found to contain 63.16 to 626.81ng/kg and 31.15 to 245.94ng/kg; lesser then the threshold levels (Mozaffari Nejad, Sabouri Ghannad *et al.* 2014). In another study aflatoxin was detected in 61.5% samples of packed and unpacked spices. Cinnamon was found to contain 0.79μg/kg; the

lease amount among all spices (Naz, Kashif *et al.* 2016). A research on 75 unpacked composite spices such as biryani, karhai, nihari, tikka, and qorma masala, was conducted to assess the event of complete aflatoxins. 77% samples found to contain aflatoxin and the level was found lesser then the allowed limit set by EU (Asghar, Zahir *et al.* 2016).

The aim of the current study was to evaluate the predominance level of aflatoxin contamination in like manner spices. 360 unpacked spice samples purchased from Karachi, Pakistan, were gone through HPLC analysis, utilizing a C18 section and Fluorescence Detector. The results will give some critical references to featuring the peril evaluation and examining the nature of spices in regards to aflatoxin contamination.

Methodology

Sample collection

During January to July 2021, a total of 360 samples of local spices samples comprising red chili, turmeric, coriander, black pepper, nutmeg, garlic and cinnamon were collected from retail shops, open bazaars and local markets of Karachi, Pakistan. A method described in AOAC method no. 977.16 was utilized to obtain homogenous samples. In brief, about 500−1000 g of each sample was collected and thoroughly mixed for 10 minutes. All samples were milled using a simple grinder to acquire a homogeneous and representative sample. An amount of 20g of each sample was taken for AFs determination. All milled samples were kept in air tight polyethylene bags at -20°C till further analysis (Asghar, Zahir *et al.* 2016).

Chemicals and Reagents

Standards of Aflatoxins G1, G2, B1 and B2 (analytical grade) were stored at 4ºC prior to use. HPLC grade methanol and acetonitrile (99.9%) were used for analysis. ASC grade glacial acetic acid, potassium chloride, potassium, sodium chloride, di-hydrogen phosphate and disodium hydrogen orthophosphate were purchased from Merck. Phosphate-buffered saline (PBS) was prepared according to procedure prescribed earlier (Sambrook 1989). 8g of NaCl was added to 0.2g of KCl, 1.44g of Na_2HPO_4 and 0.24 g of KH_2PO_4 in 1L ultrapure water and the pH was adjusted to 7.4 with HCl. Double distilled water was used for the preparation of solutions had a resistivity of >18 meqohm-cm. All other reagents were reagent grade.

Aflatoxins Extraction

20g of sample was mashed along-with 2g NaCl and extraction was carried out in 8vol/2vol methanol:water solution. The mixture was mixed in a homogenizer for 30 minutes. The filtration was carried out by Whattman No. 1 filter paper and the filtrate was diluted to six times with the addition of already prepared phosphate buffered solution (pH 7.4). Immuno affinity column with a flow rate of 2-3mL/min was used to elute. Washing of the column was carried out with 30mLdistilled water, and elution of aflatoxins with 4mL methanol. The elute was then dried at 40ºC under N_2 atmosphere. The dried residue was re-dissolved in 1mL 2:3 vol/vol mixture of methanol and water and then it was filtered through PVDF membrane having pore size 0.45µm and was stored at -18ºC until HPLC analysis.

HPLC Analysis

Reverse-phase HPLC (model LC-10ADvp solvent delivery system; auto injection, Shimadzu, Japan) C18 Brownlee reverse phase column (220x4.6mm, particle size 5μm) with C18 guard column (Perkin Elmer) was used with a fluorescence detection set at455 nm emission for aflatoxins G1 and G2 and 425nm emission for aflatoxin B1 and B2. The mobile phase was water:acetonitrile:methanol (66:17:17, v/v/v) with 4M nitric acid and 119mg/LKBr. The oven temperature was maintained to 40^{o}C with a flow rate of 1mL/min and injection volume for standard and sample extracts was kept 30μL. Since aflatoxins are possible carcinogen, care has always been practiced to avoid exposure and 10% sodium hypochlorite was used for decontamination.

Statistical Analysis

Standard deviation was estimated by using one way analysis of variance (ANOVA) according to AOAC guidelines. Calibration curves and linear regression curve showed r^2values above0.999 for each mycotoxin indicating good linearity (Williams 1984).

Result and Discussion

Validation of Method

HPLC method for the quantitative determination of aflatoxin B_1, B_2, G_1 and G_2 has been validated as described earlier (Muscarella M 2009,).

Retention Time

The retention time for Aflatoxins B_1 was obtained as 5.39minutes, for B2 10.09minutes, for G1 4.45minutes and G2 has retention time as 7.62 minutes, as shown in Figure 1.

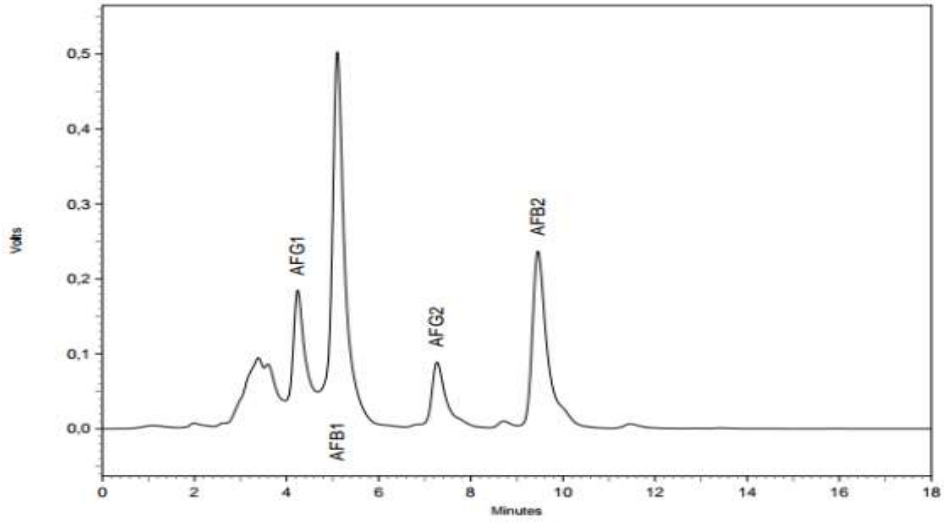

Figure 1: Retention Times of Standards of Aflatoxins B1, B2, G1 and G2

Detection Limit and Calibration Solutions

Limits of Detection LOD are the baseline to measure occurrence of aflatoxin in the spice. LOD of Aflatoxins for the spice samples was obtained and was

estimated as three times signal-to-noise ratio. The standard calibration solution of aflatoxinB1, aflatoxin G1,
aflatoxin B2 and aflatoxin G2 ranging from 0, 0.025, 0.05, 0.125, 0.25, 0.5, 1.25 ppb were prepared in 1 mL 2:3 vol/vol mixture of methanol and water and then it was filtered through PVDF membrane having pore size 0.45 µm. Seven-point calibration curve of peak versus concentration mg/L was constructed for every standard solution. Calibration curve constructed for Aflatoxin B1 is shown as Figure-2.

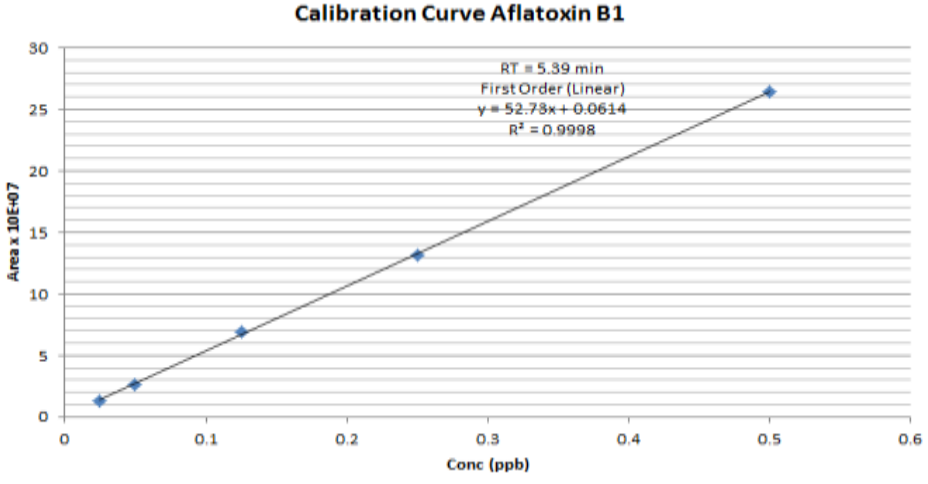

Figure 2: Calibration Curve of Aflatoxins B1

Frequency of Positive Sample Contaminated with AFB1

57 samples out of 150 were detected as positive samples of Aflatoxins B1. Samples were of Red chili (27/30), turmeric (14/20), black pepper (12/20), and coriander (4/20).

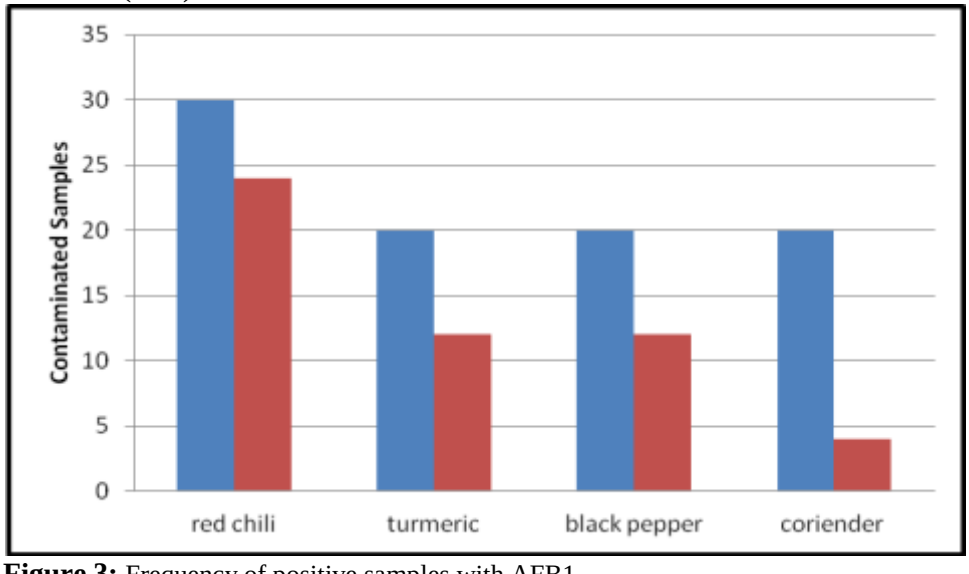

Figure 3: Frequency of positive samples with AFB1

Table 1: Aflatoxins B1 in Spices

Products	AFs	Samples Analyzed	Samples Contaminated	Positive %	Mean± SD	Concentration range (ppm)
Red Chili	B_1	30	27	90	12.5±0.17	1.56 – 15.98
Turmeric	B_1	20	14	70	14.8±0.17	8.3 – 20.62
Black Pepper	B_1	20	12	60	2.8±0.17	1.32 – 4.62
Coriander	B_1	20	4	40	0.82±0.02	0.66 – 0.98
Garlic	B_1	20	0	00	<LOD	<LOD
Nutmeg	B_1	20	0	00	<LOD	<LOD
Cinnamon	B_1	20	0	00	<LOD	<LOD

Frequency of Positive Sample Contaminated with AFB2

52 samples out of 150 were detected as positive samples of Aflatoxins B_2. Samples were of red chili (24/30), turmeric (12/20), black pepper (12/20), and coriander (4/20). However, sample of garlic, nutmeg and cinnamon were found to be AF B_2 free.

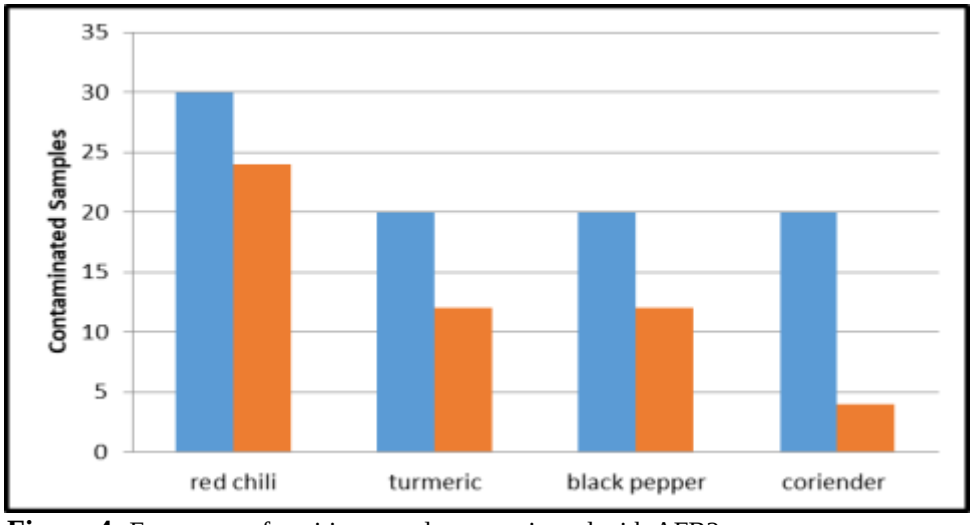

Figure 4: Frequency of positive sample contaminated with AFB2

Table 2: Aflatoxins B2 in spices

Products	Aflatoxins	Samples Analyzed	Samples Contaminated	Positive %	Mean ± SD	Concentration range (ppm)
Red chili	B_2	30	24	80	10.6± 0.09	1.12 – 12.10
Turmeric	B_2	20	12	60	11.4 ± 0.07	9.8 – 19.64
Black pepper	B_2	20	12	60	2.7 ± 0.07	1.98 – 4.64
Coriander	B_2	20	4	20	0.48 ± 0.03	0.48 – 1.0
Garlic	B_2	20	0	00	<LOD	<LOD
Nutmeg	B_2	20	0	00	<LOD	<LOD
Cinnamon	B_2	20	0	00	<LOD	<LOD

Frequency of Positive Sample Contaminated with AFG1

35 samples out of 150 i.e. red chilies (21/30), turmeric (4/20), black pepper (10/20), and coriander (4/20) were detected as positive samples for Aflatoxins G_1. However, sample of coriander, garlic, nutmeg and cinnamon were found to be AFG_1 free.

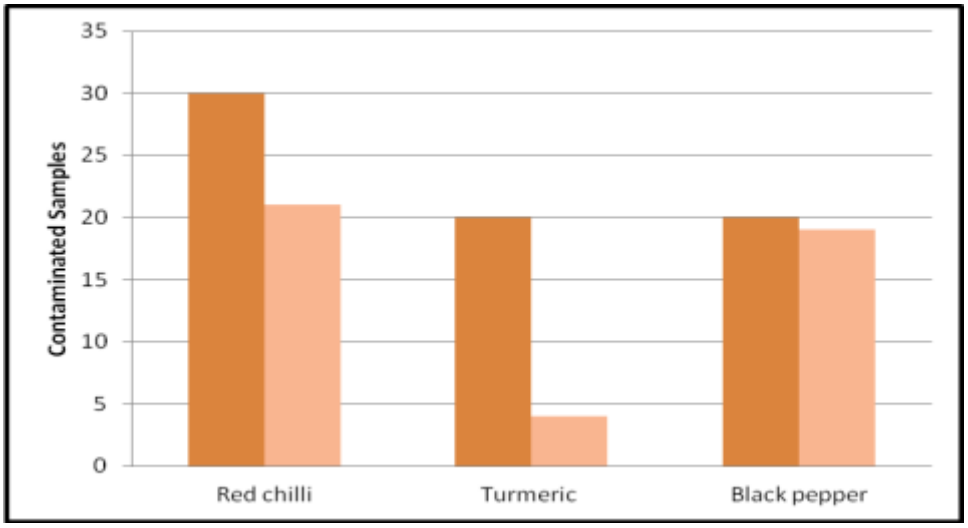

Figure 5: Frequency of positive samples of AFG1

Table 3: Aflatoxins G1 in spices

Products	Aflatoxins	Samples Analyzed	Samples Contaminated	Positive %	Mean ± SD	Concentration range (ppm)
Red chili	G_1	30	21	70	3.4 ± 0.08	1.22 – 6.46
Turmeric	G_1	20	4	20	4.8 ± 0.02	3.4 – 5.5
Black pepper	G_1	20	10	50	1.8 ± 0.02	1.42 – 2.54
Coriander	G_1	20	0	00	<LOD	<LOD
Garlic	G_1	20	0	00	<LOD	<LOD
Nutmeg	G_1	20	0	00	<LOD	<LOD
Cinnamon	G_1	20	0	00	<LOD	<LOD

Frequency of positive samples with AFG2

There were 150 samples of spices and none of the sample of spices was detected as positive in contamination with AFG_2.

Concluded Results of AFs

The concluded analytical results of occurrence of aflatoxin in 360 spices samples are summarized in Table-4

The results of current study are comparable to the study in which 75 unpacked composite spices such as biryani, karhai, nihari, tikka, and qorma masala, was conducted to assess the event of complete aflatoxins. In that study a total of 77% samples found to contain aflatoxin and the level of aflatoxin contamination in the said recipe masala was found lesser then the allowed limit set by EU (Asghar, Zahir *et al.* 2016).

Table 4: Aflatoxin B1, AflatoxinB2, AflatoxinG1 and Aflatoxin G2 in Powdered Spices

Products	Aflatoxins	Samples Analyzed	Samples Contaminated	Positive %	Mean ± SD	Concentration range (ppm)
Red Chili	B_1	30	27	90	12.5 ± 0.17	1.56 – 15.98
	B_2	30	24	80	10.6 ± 0.09	1.12 – 12.10
	G_1	30	21	70	3.4 ± 0.08	1.22 – 6.46
	G_2	30	0	00	<LOD	<LOD
Turmeric	B_1	20	14	70	14.8 ± 0.17	8.3 – 20.62
	B_2	20	12	60	11.4 ± 0.07	9.8 – 19.64
	G_1	20	4	20	4.8 ± 0.02	3.4 – 5.5
	G_2	20	0	00	<LOD	<LOD
Black Pepper	B_1	20	12	60	2.8 ± 0.17	1.32 – 4.62
	B_2	20	12	60	2.7 ± 0.07	1.98 – 4.64
	G_1	20	10	50	1.8 ± 0.02	1.42 – 2.54
	G_2	20	0	00	<LOD	<LOD
Coriander	B_1	20	4	40	0.82 ± 0.02	0.66 – 0.98
	B_2	20	4	20	0.48 ± 0.03	0.48 – 1.0
	G_1	20	0	00	<LOD	<LOD
	G_2	20	0	00	<LOD	<LOD

Conclusion

Occurrence of aflatoxin was detected for the most of the samples evaluated in the current study. A total of 360 samples of spices were tested for each aflatoxin. Out of which 57 were detected as positive for aflatoxin B_1, 52 samples were found to be contaminated with AF B_2, 35 samples of spices were being tested as positive with AF G_1. Whereas Aflatoxin G_2 was found to be absent in all samples. Maximum level of aflatoxin $B_1$was detected as 8.3 – 20.62ppm in turmeric and minimum level was found in coriander that is 0.66 – 0.98ppm. Maximum level of aflatoxin B_2 was detected as 9.8 – 19.64ppmin turmeric and minimum level was found in coriander that is 0.48 – 1.0ppm. Maximum level of aflatoxin $B_1$was detected as 1.22 – 6.46 in red chili and minimum level was found in black pepper that is 1.42 – 2.54ppm.

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