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RESEARCH PAPER

Assessment of aflatoxin B_1 content and aflatoxigenic molds in imported food commodities in Muscat, Oman

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تقييم محتوى الأفلاتوكسين B_1 والفطريات المنتجة لأفلاتوكسين في السلع الغذائية المستوردة في مسقط بسلطنة عمان

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Abstract. Aflatoxins, mainly produced by *Aspergillus flavus* and *A. parasiticus* are considered as serious food safety and human health issues due to their hepatotoxic effects. In the present study, the occurrence of aflatoxin B1 (AFB1), the most potent human liver carcinogen, and prevalence of toxigenic isolates of *Aspergillus* spp. were assessed in 140 food commodities in Muscat markets, Oman, and the 95 quarantined imported food commodities. These samples consisted of rice, corn, peanut, red chilli powder, soybean, dried dates and tree nuts. AFB1 was analyzed using competitive ELISA/LC-MS and the aflatoxigenic fungi were detected using plating technique followed by molecular identification. No AFB1 was detected in 89 (63.6%) samples collected from local markets, while 44 (31.4%) samples contained 1-5 ppb and the remaining 7 (5%) samples (red chili powder) contained 6-10 ppb. None of the samples exceeded the maximum permissible limit of 10 ppb set for foods by Oman legislation. Of the 95 quarantined samples, only 17 (17.9%) samples were positive and contained AFB1 at concentrations ranging from 1-3.4 ppb. Four isolates of *Aspergillus* pp. were isolated from the collected samples and were identified as *Aspergillus flavus* (A14, A16 and A23) and *A. chevalieri* (A46) on the basis of internal transcribed spacer (ITS) sequences of ribosomal DNA. Among them, *A. flavus* strain A14 alone produced AFB1 (7.6 ppb), while A16, A23, and A46 were non-toxigenic. This is the first detailed report on the occurrence of AFB1 in food commodities imported into Oman.

Keywords: Aflatoxin B1; aflatoxigenic fungi; *Aspergillus flavus*; food-borne mycotoxins

المستخلص: تعتبر الأفلاتوكسينات و التي تنتج بشكل رئيسي من اسبراجليس فلافوس و اسبراجليس باراسيتيكس، من المشاكل الخطيرة لسلامة الأغذية وصحة الإنسان بسبب آثارها السامة على الكبد. في هذه الدراسة ، تم تحديد الأفلاتوكسين B1 (AFB1) من نوع الاسبرجليس ، المسبب لسرطان الكبد البشري واشدها فعالية ومنتجة للسموم في ١٤٠ سلعة غذائية في أسواق مسقط بسلطنة عمان و ٩٥ سلعة غذائية مستوردة خاضعة للحجر الصحي. اشتملت هذه السلع على الأرز والذرة والفول السوداني ومسحوق الفلفل الأحمر وفول الصويا والتمور المجففة والمكسرات. تم تحديد AFB1 باستخدام التقنية الحديثة في المقايسة الأمتصاصية المناعية للانزيم المرتبط و الكروماتوجرافيا السائلة المقترنة بمطياف الكتلة LC–MS ELISA / وتم تحديد الفطريات المنتجة للسموم بالتعريف الجزيئي. لم يوجد AFB1 في ٨٩ (٦٣,٦٪) عينة تم جمعها من الأسواق المحلية ، بينما احتوت ٤٤ (٣١,٤٪) عينة على ١–٥ جزء في البليون ، واحتوت ٧ عينات (٥٪) المتبقية (مسحوق الفلفل الأحمر) على ٦–١٠ جزء في البليون. لم تتجاوز أي من العينات الحد الأقصى المسموح به للأغذية حسب التشريعات العمانية وهو ١٠ أجزاء من البليون. من ٩٥ عينة محجورة ، كانت ١٧ (١٧,٩٪) عينة فقط إيجابية واحتوت على AFB1 بتركيزات تتراوح بين ١–٣,٤ جزء في البليون. تم عزل أربع انواع من الاسبراجليس من العينات التي تم جمعها وتم تحديدها على أنها اسبراجليس فلافوس (A16، A14 و A23) و (A46) واسبراجليس شيفالييري على اساس منطقة التمييز الحمض النووي (ITS) للحمض النووي الريبوزومي. من تلك الانواع ، أنتجت سلالة A14 من اسبراجليس فلافوس وحدها AFB1 في حدود ٧,٦ جزء في البليون ، في حين أن A16 و A23 و A46 كانت غيرمنتجة للسموم. هذا هو أول تقرير مفصل عن وجود AFB1 في السلع الغذائية المستوردة في عمان.

الكلمات المفتاحية: الافلاتوكسين B1، الفطريات المنتجة للسموم، اسبراجليس فلافوس ، السموم الفطرية التي تنقلها الأغذية.

Introduction

Aflatoxin contamination in food products poses a serious threat to human health as aflatoxins are carcinogenic, teratogenic and immunosuppressive chemical compounds (Benkerroum, 2020). Aflatoxin has been proven to be genotoxic and neurotoxic in test animals (Linardaki et al., 2017). Aflatoxins are produced predominantly by the filamentous *Aspergillus flavus* and *A. parasiticus* as secondary metabolites (Khan et al., 2021). These toxigenic molds contaminate various agricultural commodities at pre-harvest stage in the field, or during post-harvest drying/curing and storage and secrete aflatoxins through the process called "exocytosis" (Chanda et al., 2010). Aflatoxin contamination has been reported in several agricultural commodities such as corn, peanut, rice, chilli, cottonseed, cashew, pistachios, hazelnuts, almonds, cocoa beans, soybeans, dried figs and date fruits (Adeyeye, 2016; Gallo et al., 2021; Kumar et al., 2021)

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Currently 20 different forms of aflatoxins have been characterized among which aflatoxin B1, B2, G1 and G2 are commonly found in agricultural commodities (Kumar et al., 2017). Among the aflatoxins, AFB1 is the most ubiquitous and threatening mycotoxin to humans and considered as one of the most potent naturally occurring hepato-carcinogens known. When cow, camel, goat or other lactating animal consumes aflatoxins B1 contaminated feed, aflatoxin M1 will be formed as a result of the metabolic process in the liver of animals and excreted in milk (Sharma et al., 2020). Aflatoxin M1 in camel milk samples collected from Jeddah, Saudi Arabia (Bokhari et al., 2017) and Abu Dhabi, United Arab Emirates (Saad et al., 1989) was reported, whereas, the presence of AFB1 in the meat and eggs of poultry birds fed with aflatoxin-contaminated feed has been reported (Trucksess et al., 1983).

Ingestion of food or feed contaminated with excessive levels of aflatoxins leads to the disease called "aflatoxicosis" in humans and animals (Williams et al., 2004; Ogodo and Ugbogu, 2016; Sarma et al., 2017). The World Health Organization (WHO) ranked the aflatoxicosis as the 6th among the top 10 important health risks to human beings (Williams et al., 2004). The International Agency for Research on Cancer (IARC) classified aflatoxins as class I human carcinogens (International Agency for Research on Cancer, 2002). In humans, acute and chronic exposures to aflatoxins cause a variety of clinical symptoms including liver damage, pulmonary edema, haemorrhage and in some cases death (Wu et al., 2014; Sarma et al., 2017).

The existence of aflatoxins in the food chain threatens people's livelihood, their health and food security (Kumar et al., 2017). Hence, over 100 countries enforce aflatoxin tolerance levels in foods (van Egmond et al., 2007). The Food and Drug Administration (FDA) of the United States of America fixed an aflatoxin tolerance limit of 20 parts per billion (ppb) for foods. The Sultanate of Oman has fixed the maximum permissible level of aflatoxin B1 in foodstuffs at 10 ppb (Agenda Item 11, Codex committee on contaminants in foods, Eleventh Session, Rio De Janeiro, Brazil, 3-7 April 2017). Oman imports most of the food grains from international markets (Mbaga, 2013). However, information on the aflatoxin contents, precisely the carcinogenic type AFB1, in food commodities in Oman is limited. In this study corn, peanut, red chilli powder, rice, soybeans, dates and tree nuts samples collected from local markets, super markets and Quarantine department, Ministry of Agriculture, Fisheries and Water Resources, Sultanate of Oman were assessed for the presence of AFB1. Furthermore, the prevalence of aflatoxigenic strains of *Aspergillus* spp. in the collected samples was determined.

Materials and Methods

Sample collection

A total of 140 samples (100-1000 g) were collected from the retail shops and supermarkets in Oman in 2020. The collected samples consisted of raw peanuts (15), roasted peanuts (5), walnuts (15), raw pistachios (18), roasted pistachios (5), raw almonds (14), roasted almonds (3), rice (8), corn (8), red chili powder (16), dried dates (6), cashew (16), roasted cashew (8), hazelnut (1), peacon (1) and Brazil nut (1). These food commodities were precisely selected due to their high frequency consumption in most Arabian Gulf countries including Oman. The collected samples were kept in sterile bags and stored at 4 °C until analysis.

Another 95 imported food commodities were collected from the Quarantine Department, Ministry of Agriculture, Fisheries and Water Resources, Sultanate of Oman during July - August 2021. These samples consisted of rice, peanut, almonds, pistachios, cashew and soybeans. The collected samples were kept in sterile bags and stored at 4°C until analysis. Quarantined foods were selected to tackle the source of AFB1.

Determination of aflatoxin B1

The samples were ground into fine powders by using a blender and 5 g of powdered sample was added with 25 ml of 70% methanol and mixed well by vortexing. The homogenate was centrifuged at 10000×g for 10 min at room temperature (25±2°C) and the supernatant was collected. The supernatant was diluted with distilled water (1:1, v/v) and AFB1 content in the samples were analyzed using a commercial enzyme-linked immunosorbent assay (ELISA) kit (RIDASCREEN Aflatoxin B1; R-Biopharm AG, Darmstadt, Germany) according to the manufacturer's instructions.

Isolation of *Aspergillus* spp.

Aspergillus spp. were isolated from the collected samples on potato dextrose agar (PDA) (Oxoid, UK) medium under laboratory conditions (Karthikeyan et al., 2009).

Molecular identification of *Aspergillus* spp.

Genomic DNA was isolated from the 7-day-old fungal mycelia as described by Karthikeyan et al. (2009). The Internal Transcribed Spacer (ITS) regions of the fungal rDNA were amplified by PCR by using the extracted DNA and ITS4 and ITS5 primers (White et al., 1990) as described by Halo et al. (2018). The PCR products of the expected size were sequenced (Macrogen, Korea) and the sequences were subjected to BLAST searches using the National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov).

Assessment of toxigenic potential of *Aspergillus* spp.

The mold cultures were grown in 250 ml conical flasks containing 100 ml of SMKY medium (200 g sucrose, 0.5 g $MgSO_4 7H_2O$, 0.3 g KNO_3 and 7 g yeast extract in 1l of distilled water) at 25±2 °C for two weeks. At the end of incubation period, the culture filtrates were collected by filtration using Whatman No. 1 filter paper. The culture filtrate (500 µl) was extracted with chloroform (1:1 v/v) and the chloroform fraction was fully evaporated using a water bath at 60°C. The residue was dissolved in 20 µl of HPLC-grade methanol and analyzed by Agilent LC/MS/MS, equipped with high-performance autosampler (G4226A), quaternary pump (G4204A), thermostatted column compartment (G1316C) and 6460 Triple Quad MS detector. Chromatographic separations were performed with Symmetry C8 5 µm, 3 mm × 150 mm column (Waters). The column was maintained at 45°C. The injection volume was 5 µl. The mobile phase used was acetonitrile (eluent A) and water (eluent B), both with 0.1% formic acid under gradient condition (eluent A 10-70% in 0-1 min, 70-95% in 1-2 min, hold at 95% for 4 min (2-6 min), 95-70% in 6-7.5 min, 70-10% in 7.5-8 min and hold at 10% for 1 min) with a flow rate of 0.3 ml per min. The following MS detector parameters were used for the analysis: gas temperature 300°C, gas flow 3 L min^{-1}, nebulizer pressure 50 PSI, sheath gas heater 375°C, sheath gas flow 10 L min^{-1}, capillary voltage 3500 V, scan range 100 to 3000 *m/z* and positive polarity. Mass spectra data were acquired and processed by Agilent MassHunter workstation and Agilent MassHunter qualitative analysis software, respectively.

Results and Discussion

Out of 140 food commodities from local markets and supermarkets analyzed, AFB1 has detected in 51 (36.4%) samples (Table 1). However, AFB1 content in all the food samples was below the maximum tolerance limit (10 ppb) set by Oman legislation. The levels of AFB1 in 7 red chilli powder samples exceeded the maximum tolerance limit of 5 ppb set by the European Union for chilli (Ezekiel et al., 2019; Gallo et al., 2021). The low levels of AFB1 contamination in agricultural commodities might be due to good agricultural and storage practices. Reasonably high AFB1 content (above 5 ppb) in red chilli powder suggests that the contaminated red chilli may be a major source of consumer exposure to aflatoxins in Oman. Chilli is considered as one of the favourable substrates for the growth of many agronomically important molds including *A. flavus* and toxin production (Supriya et al., 2015). The presence of mycotoxins such as aflatoxins, ochratoxin A, fumonisins, zearalenone and patulin in capsicum has been documented (Costa et al., 2019; Chuaysrinule et al., 2020). Moreover, Iqbal et al. (2010) reported that more than 50% of chilli pepper samples from Punjab region of Pakistan contained above 5 ppb of AFB1. Chuaysrinule et al. (2020) reported that 96.7% of dry chilli pods and 86.7% of chilli powder collected from markets in Thailand were contaminated with molds; among them *Aspergillus* section *Flavi* were predominant. Kiran et al. (2005) reported that chilli pods kept in cold storage were contaminated with aflatoxin B1 to the extent of 5.5 ppb. Paterson (2007) recorded up to 93 ppb of AFB1 in dry chilli samples obtained from markets in Pakistan. Ezekiel et al. (2019) reported that 69% of the chili pepper samples collected from local markets and farmers' stores in Nigeria were contaminated with aflatoxins with an average of 8.9 ppb. Of the 95 food samples obtained directly from the Quarantine department and evaluated, 17 samples were found to be positive for AFB1 but the levels were below 5 ppb (1-3.4 ppb) (Table 2). This is probably due to adoption of strict plant quarantine regulations and phytosanitary measures.

The water content of chilli at the time of harvest is usually between 65% and 80% [water activity (aw) 0.995]. In order to minimize mold growth and mycotoxin production, the water content of the harvested chilli should be reduced quickly to 13% (a_w 0.50) by drying (Toontom et al., 2012; Sanzani et al., 2016). As the dried chilli is highly hygroscopic, they need to be properly packed soon after drying (Costa et al., 2019). At retail markets, rehydration of dried chilli may favour growth of molds and mycotoxin production. Sahar et al. (2015) observed a direct relationship between aflatoxin content in red chilli and moisture content, thus drying of chilli to less than 10% moisture content to prevent mold growth and to minimize aflatoxin contamination was recommended. Hence, proper drying and storage of dry chilli in retail shops may help in preventing mold growth and aflatoxin production.

Of the 235 samples tested, only 4 samples viz., A14 (from cashew), A16 (from peanut), A23 (from peanut) and A46 (from corn) collected from local shops showed the presence of *Aspergillus* spp. None of the samples obtained from the quarantine department showed the presence of *Aspergillus* sp. The mold isolates were found to be *A. flavus* (A14, A16, A23; 100% identity) and *A. chevalieri* (A46; 100% identity) based on the sequences of the ITS regions of rDNA. The sequences were deposited to the GenBank with the accession numbers MW386304; MW386306; MW386307 and MW386308. The natural occurrence of aflatoxin-producing molds in chilli has been reported (Jeswal and Kumar, 2015; Chuaysrinule et al., 2020). The absence of aflatoxin-producing molds in other samples in this study does not guarantee that those are free of aflatoxins (Pitt et al., 2000). For instance, the 7 red chilli samples that showed AFB1 above 5 ppb were free from *Aspergillus* spp. This might be due to removal of toxigenic fungi from red chilli during postharvest processing or disappearance of molds after secretion of the toxins in the substrate.

Among the 4 strains evaluated for *in vitro* production of AFB1, *A. flavus* strain A14 was found to be capable of producing AFB1 and the amount of AFB1 produced

Table 1. Aflatoxin B1 content in food commodities in Muscat markets, Oman

Food commodities	Total number of samples	Number of samples and AFB1 concentration range (ppb)					
		0	1-5	6-10	11-15	16-20	Above 20
Raw Peanuts	15	9 (0)	6 (1.0-4.4)	-	-	-	-
Roasted Peanuts	5	4 (0)	1 (1.8)	-	-	-	-
Walnuts	15	3 (0)	12 (1.0-4.8)	-	-	-	-
Raw Pistachios	18	13 (0)	5 (1.0-1.6)	-	-	-	-
Roasted Pistachios	5	5 (0)	-	-	-	-	-
Raw Almonds	14	12 (0)	2 (1.0-1.4)	-	-	-	-
Roasted Almonds	3	3 (0)	-	-	-	-	-
Rice	8	6 (0)	2 (1.6-2.4)	-	-	-	-
Corn	8	6 (0)	2 (1.2-2.0)	-	-	-	-
Red Chilli powder	16	-	9 (2.8-4.8)	7 (7.4-9.8)	-	-	-
Dried Dates	6	6 (0)	-	-	-	-	-
Cashew	16	14 (0)	2 (1.2)	-	-	-	-
Roasted Cashew	8	6 (0)	2 (1.0)	-	-	-	-
Hazelnut	1	1 (0)	-	-	-	-	-
Pecan	1	-	1 (3.8)	-	-	-	-
Brazil nut	1	1 (0)	-	-	-	-	-
Total	140	89	44	7			

was 7.6 ppb, while A16, A23, and A46 were non-toxigenic. The contamination of red chilli depends on the virulence and toxigenic potential of the *Aspergillus* spp. strains. Several studies found no correlation between the level of AFB1 in foods and the population of *A. flavus* as toxin production is governed by genetic make-up of mold species, availability of nutrients in the substrate, water activity, temperature, etc. (Paterson, 2007; Santos et al., 2011). Though *A. flavus* strain A14 (aflatoxigenic) was isolated from cashew in this study, no AFB1 was detected in this sample. Elshafi et al. (2002) found that 9 out of 20 (45%) isolates of *A. flavus* isolated from spices in Oman produced aflatoxins (50-90 ppb). Jeswal and Kumar (2015) reported that 56% of *A. flavus* isolated from red chilli were toxigenic and produced aflatoxins. Gherbawy et al. (2015) while studying the mycobiota of chilli products in Saudi Arabia reported that some *Aspergillus* strains did not produce aflatoxins, though they had the gene clusters for aflatoxin biosynthesis.

Conclusion

In this work, we found that AFB1 content in all the food samples commercialized in Muscat, Oman, was within the permissible limit set by Oman Regulations. Only red chilli powder samples (5%) were found contaminated with AFB1 above 5 ppb, the maximum tolerance limit fixed by the European Union. Such aflatoxins contaminated chilli may be a potential route of exposure of local people to aflatoxins. The levels of AFB1 in the food commodities obtained directly from the port of entry through the quarantine department were less than 5 ppb. The occurrence of an aflatoxigenic strain of *A. flavus* in one of the samples indicates a possible risk of aflatoxin production in foods especially if stored under improper conditions. Our results suggest that proper storage practices need to be followed in the retail shops/processing units to prevent mold growth and AFB1 contamination. Further studies are needed to increase sampling

Table 2. Aflatoxin B1 contamination in food commodities imported (from July 2021 to August 2021) in Oman

Food commodities	Total number of samples	Number of samples and AFB1 concentration range (ppb)					
		0	1-5	6-10	11-15	16-20	Above 20
Almond	1	1 (0)	-	-	-	-	-
Pistachios	1	1 (0)	-	-	-	-	-
Cashew	3	2 (0)	1 (1.0)	-	-	-	-
Rice	71	58 (0)	13 (1.0-3.4)	-	-	-	-
Soybean	17	14 (0)	3 (1.0-1.8)	-	-	-	-
Peanut	2	2 (0)	-	-	-	-	-
Total	95	78	17				

size and analyze other food-borne mycotoxins, such as AFB2, AFG1, AFG2 and ochratoxin A in the chilli samples. To our knowledge, this is the initial detailed report on the levels of AFB1 contamination in foods in Oman.

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