Toxigenic fungi and aflatoxin associated to nuts in Saudi Arabia

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Abstract: A survey was carried out in July-Sept., 2009 to obtain data on the occurrence of aflatoxin and the aflatoxins-producing potential of fungi isolated from nuts (almonds, peanuts, hazelnuts, pistachio nuts, Walnut and Cashew) in region of Qassim in Saudi Arabia. The samples were analyzed for aflatoxins by immune affinity column (IAC) clean-up with liquid chromatography and fluorescence detection. Percentages of positive samples with aflatoxins were 80, 80, 60, 40, 40, 20 % for pistachio, peanuts, walnuts, almonds, hazelnuts and cashews. Concentrations of aflatoxin B_1 were ranged between (38- 45, 11- 90, 41 -90, 0.3-3.6 ,62-120 and 70-140 " µg/kg") respectively.100% of samples showed variable incidence of fungal contamination. Fungi present in samples were *Penicillium spp, Aspergillus niger*, *A. flavus* and *Rhizopus spp*. Results showed that isolates of *A. flavus* were able to produce aflatoxins B_1, B_2, G_1, and G_2. The purpose of the survey was to determine levels of aflatoxins and to monitor the effectiveness of the controls in place to limit consumer exposure to aflatoxins.

Keywords: exposure to carcinogens, aflatoxins, nuts, HPLC, Fungi, food contaminants**.**

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1. Introduction

Mycotoxins; toxic secondary metabolites of fungi are biological in origin. Despite efforts to control fungal contamination, toxigenic fungi are ubiquitous in nature and occur regularly in worldwide food supplies due to mold infestation of susceptible agricultural products, such as cereal grains, nuts, and fruits. Thousands of mycotoxins exist, but only a few present significant food safety challenges. The natural fungal flora associated with foods is dominated by three genera of fungi; *Aspergillus*, *Fusarium*, and *Penicillium,* which except for the *Fusarium* plant pathogens, may include commensals as well as pathogens. Groundnut is frequently infected with fungi that produce mycotoxins during and after harvesting, which affect the quality and safety of human food (Martin *et al*., 1999). They are toxic to humans and animals, cause significant reductions in crop yield and cause economic losses (Gourama and Bullerman, 1995; Gqaleni *et al.,* 1996). Their occurrence in various countries has been well documented (Bathnagar and Garcıa, 2001). *Aspergillus flavus* and *Aspergillus parasiticus* are important contaminants of certain foods and animal feeds because of their ability to produce aflatoxins (Farr *et al.,* 1989). When these fungi invade and grow in commodities such as peanuts, corn and cottonseed, the resulting contamination with aflatoxins often makes the commodities unfit for consumption (Vardon, 2003). Aflatoxins are considered the most carcinogenic, mutagenic and teratogenic substances found naturally in foods and feeds (Conner, 1993). These metabolites cause liver damage to humans and to most experimental animal species tested (Gradelet *et al.,* 1997). Consumption of mycotoxin-contaminated foods has been associated with several cases of human poisoning, or mycotoxicosis, sometimes resulting in death (Bathnagar and Garcıa, 2001). The International Agency for Research on Cancer (IARC) has classified aflatoxin B_1 (AFB_1) as a group I carcinogen, primarily affecting liver (IARC, 1993). Natural occurrence of AF in nuts has been studied in various countries. According to a report from Mexico, 2.2% of pistachio nut samples analyzed contained AF higher than 20 ng/g (JECFA, 1998). In Sweden, 9.5% pistachio nut samples contained AFB1 higher than 2 ng/g (Thuvander *et al.,* 2001). According to Ministry of Agriculture and Rural Affairs, Republic of Turkey, (2002) analysis of 523 pistachio nut samples in Turkey the mean of AFB1 ranged 1–3.78 ng/g and the maximum level detected was 113 ng/g.

The present study was undertaken to determine the occurrence of fungi, can production of aflatoxins in nuts in Saudi Arabia.

2. Material and Methods

Methods:

Samples:

Thirty samples of nuts (almonds, hazelnuts, pistachio nuts, Cashew ,Walnut and peanuts) were randomly collected from Saudi Arabia , Qassim region (Buraydah, Unayzah, Al-Ras, Al- Badaye and Al- Bukayriyah) during Juli to Sept at 2009.

Isolation of fungi associated with nuts.

Fungi associated with nuts were isolated according to methods of Lichtwardth *et al.*, (1958), Mislivec (1977) and Ichinoe *et al.* (1983). All nut samples were immersed in sodium hypochlorite solution (5%) as a sterilizer, for 3 minutes, rinsed 3 times in sterile distilled water then dried between sterile filter paper. Isolation of fungi was made by randomly taking 100 disinfested grains from samples of each location and directly plated in 20 Petri-dishes (5 grains per dish) on Czapek's agar medium, then incubated at 25°C for 4 days. Then, the percentage of the infected grains was determined and the fungal colonies that developed from the infected grains were counted, isolated, purified and maintained on a slant potato dextrose agar (PDA) medium for identification trials.

Identification of the fungal isolates:

All the isolated fungi were identified by studying the cultural characteristics, as well as the microsocial structures on Gzapek's agar medium, according to Harigan and Margaret (1966), and according to the procedures of Gilman (1957), Barnnett and Hunter (1972) Numbers of the species of *Aspergillus* were classified according to the key published by Raper and Fennell (1965), while for species of other isolated fungi the "Manual of clinical mycology" by Count *et al.* (1954) was used.

Production of AFs by isolated *A. flavus* strain:

The ability of AFs production by *A. flavus* strains using liquid media (YES) was investigated according to Singh *et al.,* (1991).

Aflatoxin standard:

Preparation of aflatoxin standard was carried out according to the Association of Official Analytical Chemists, A.O.A.C.(2000). Crystals of aflatoxins B_1, B_2, G_1 and G_2 were diluted using benzene-acetonitrile (98: 2 v/v) to obtain a concentration of 8 to 10 µg/ml (stock solution).

Equipment and chromatographic conditions:

Extraction procedure:

AFs were analyzed in nuts according to the method reported by Stroka, et al., (2000). Briefly, 10 g of nut samples were added to 1 g of NaCl , then blended with 40 ml of methanol/water (80:20) and 20 ml of cyclohexane for 3 min. After separation of the two phases, cyclohexane was eliminated. Extracts were filtrated on a Whatman filter paper No. 4. Immunoaffinity column clean-up An aliquot of 10 ml was diluted with 60 ml of PBS buffer (pH 7.4). An immunoaffinity column (IAC AflaTest, Vicam, USA) for AFs analysis was conditioned with 10 ml of PBS buffer by gentle syringe pressure at a flow rate of 5 ml/min. Then, the mixture of the filtrate diluted extract (70 ml) was applied to the IAC column (1–2 drops per second), followed by a washing with 20 ml of bi-distilled water and then dried with air. AFs were then slowly eluted from the IAC with 2 ml methanol into a glass vial.

Precolumn derivatization:

The eluate was evaporated to dryness with a gentle stream of N2 at 52 ºC, redissolved with 100 µl of TFA for 3 min, re-evaporated to dryness with N2 at 52□ C, and reconstituted in 500 µl of the mobile phase for LC analysis. TFA was added to AFs working standards in the same conditions as the extract samples to derivatize AFB1 , AFB2 , AFG1and AFG2.

Determination of Aflatoxins A by HPLC:

High-performance Liquid Chromatography "HPLC" (Agilent 1100 series) equipped with a fluorescence detector (G 1321A) analysis was carried out with a liquid chromatograph equipped with solvent delivery systems (Agilent Technologies, Inc. 200 Regency Forest Drive, Suite 330 Cary, NC 27511 USA) system containing a G1322A Vacuum Degasser, a G1312A Binary pump and a reverse-phase analytical column packed with C_{18} material (Agilent ZORBAX, DB- 5 µm, 150 mm × 4.6 mm) . The mobile phase consisted of water: acetonitril : methanol (240:120:40) (Deabes, 2008). Separation was performed at 40 °C temperature at a flow rate of 1.0 ml min-1; the injection volume was 50 µl for both standard solutions and sample extracts by auto sampler (G1329A). The detection was performed using fluorescence detector was operated at an excitation wave length of 360 nm and an emission wave length of 440 nm .

Quantitation*:* The mixed solutions of standard as well as sample extract after derivatisation were filtered through a 0.22 µm membrane filter and loaded (50 µl) into autosampler. The elution order of the four aflatoxins was G_2, B_2, G_{2a} (G_1 derivative), B_{2a} (B_1 derivative). AFs contents in samples were calculated from chromatographic peak areas using the standard curve.

3. Results and Discussion:

Isolation and identification of fungi associated with nuts:

It is well known that some fungi grow on/ and in nuts and these fungi can deteriorate the stored nuts. Therefore, the present work, was started by isolation and identification such fungal species. Isolation of fungi was made by using Czapek's agar as growing media.

Fungi isolation on Czapek's agar medium:

Data in Table (1) represent the fungi associated with Czapek's agar medium from nuts purchased from retail markets in Qassim, Saudi Arabia during the summer season of 2009.

Table (1): Fungi associated with nut samples collected from Qassim region Saudi Arabia during 2009.

Commodities	No of samples	% of Infections	Total fungal Counts	Isolated Fungi		
				Aspergilluss	*Penicillum*	*Rhizopus*
Almond	**5**	**100**	**120**	**57.00**	**40.00**	**3.00**
Pistachio	**5**	**100**	**153**	**60.00**	**32.00**	**8.00**
Hazelnut	**5**	**100**	**140**	**53.00**	**33.00**	**14.00**
Cashew	**5**	**100**	**60**	**50.00**	**40.00**	**10.00**
Walnuts	**5**	**100**	**160**	**57.00**	**33.00**	**10.00**
Peanuts	**5**	**100**	**163**	**67.00**	**24.00**	**9.00**

Table (2): Toxicity of *Aspergillus flavus* isolated from nuts and the ability for aflatoxins production in YES medium.

Kind of Nuts	Isolates of *Aspergillus* tested (No.)	Isolates of *A. flavus* (No.)	Isolates of *A .flavus* producing Aflatoxins (No.)	Isolates of *A. flavus* producing Aflatoxins (%)	Total Aflatoxins concentration mg/l	
					Min	Max
Almonds	40	25	22	88	20	40
Pistachio	60	42	33	78	12	60
Hazelnuts	65	32	27	84	16	50
Cashews	30	15	7	46	1.2	6
Walnuts	70	50	40	80	30	70
Peanuts	90	62	58	93	65	150

Table (3): Survey of aflatoxins in nuts collected from Qassim, Saudi Arabia during summer , 2009

Kind of nuts	No. of samples	No. of positive samples	% of positive samples	Aflatoxins concentration µg/kg							
				AFB1		AFB2		AFG1		AFG2	
				Min	Max	Min	Max	Min	Max	Min	Max
Almonds	5	2	40	38.00	45.00	11.00	13.00	0.00	2.30	0.00	4.20
Pistachio	5	4	80	11.00	90.00	2.00	70.00	1.10	55.00	1.90	25.00
Hazelnuts	5	2	40	41.00	55.00	12.90	18.00	3.10	6.00	5.20	8.40
Cashews	5	1	20	0.30	3.60	0.10	2.30	0.00	0.00	0.00	0.00
Walnuts	5	3	60	62.00	120.00	13.00	33.00	5.30	11.00	7.90	17.00
Peanuts	5	4	80	70.00	140.00	18.00	71.00	2.20	10.10	3.10	13.40

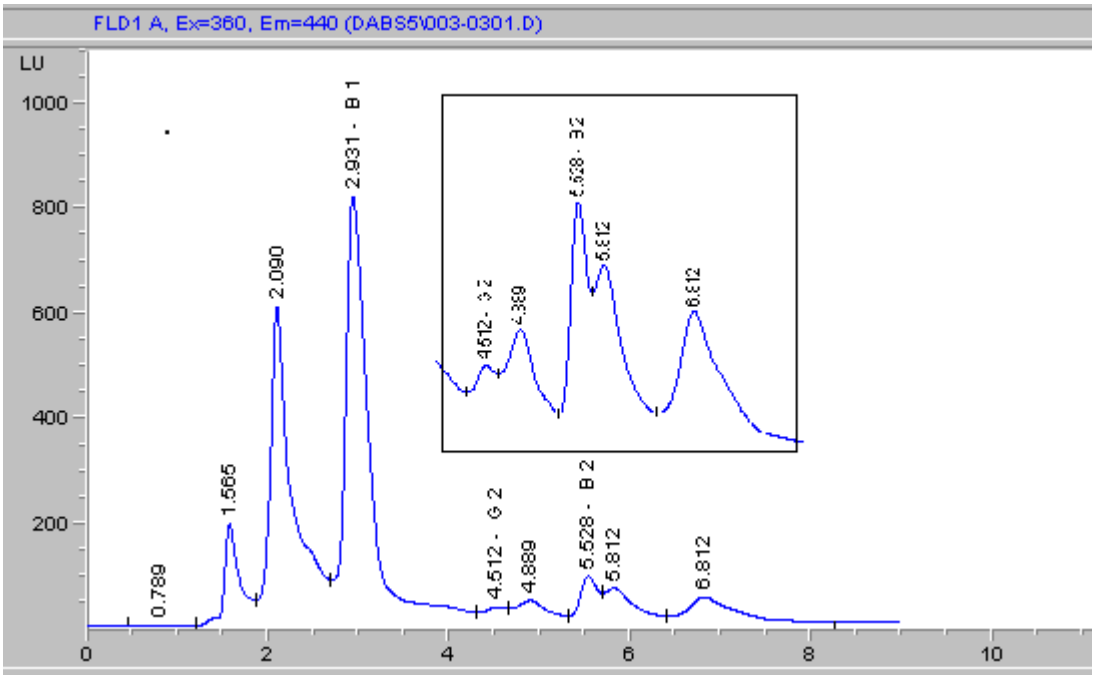

Figure (1): High Performance Liquid Chromatogram of a positive sample of aflatoxins (AFG_1,AFB_1, AFG_2 and AFB_2)

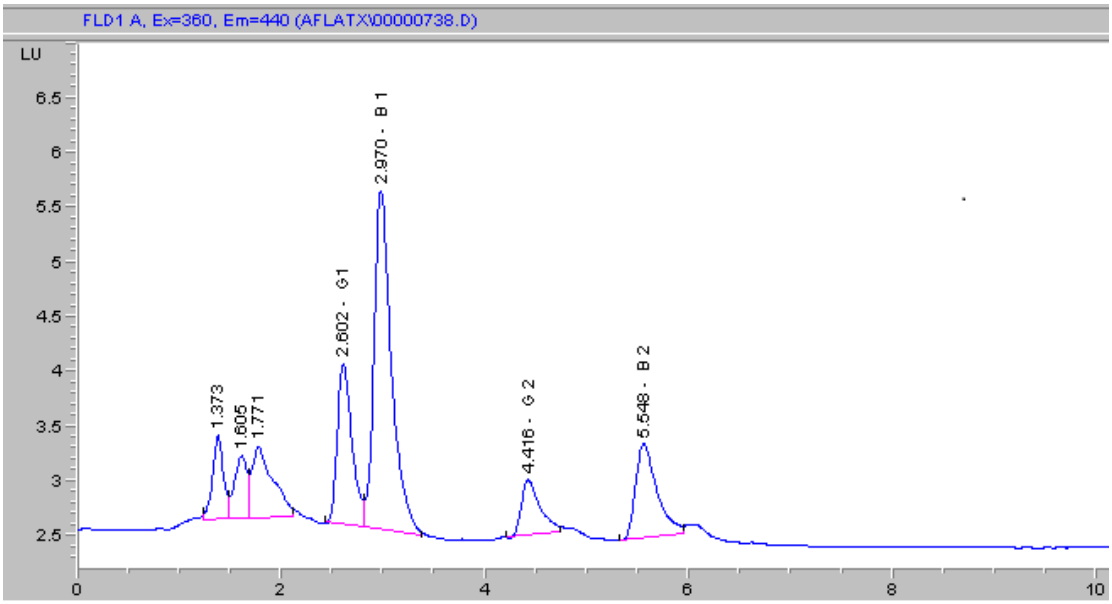

Figure (2): High Performance Liquid Chromatogram of a standard of aflatoxins (AFG_1,AFB_1, AFG_2 and AFB_2)

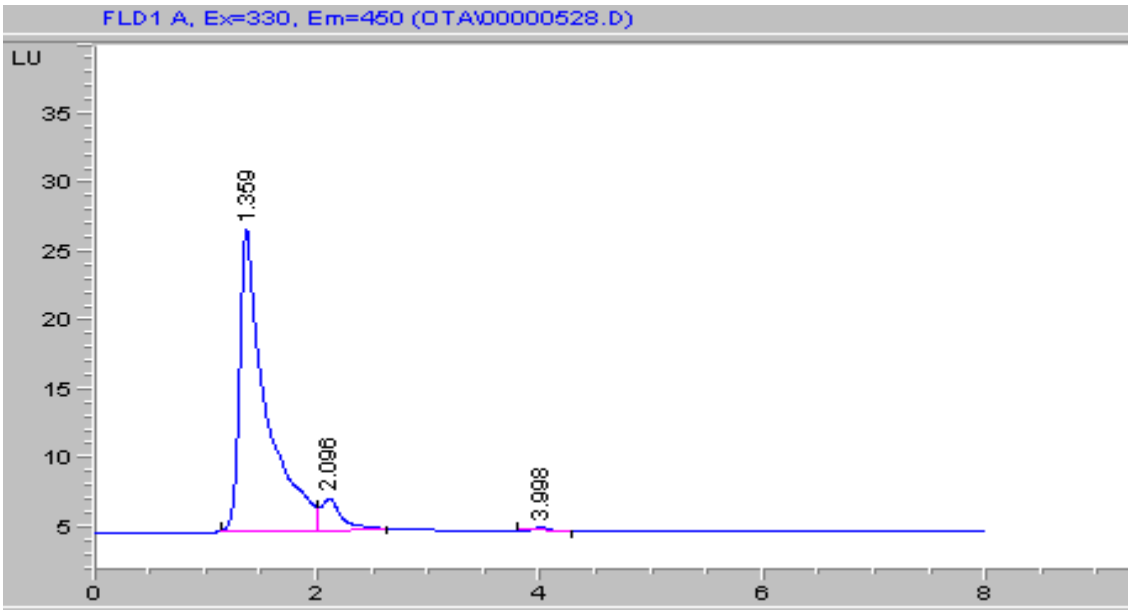

Figure (3): High Performance Liquid Chromatogram of a negative sample of aflatoxins (AFG_1,AFB_1, AFG_2 and AFB_2)

Data in Table (1) showed the percentage of infected nuts kernel and the percentage of the fungal genera which isolated in summer of 2009. Data revealed that, the percentage of fungal infected samples was 100% in all nuts samples collected from retail markets in Qassim region . The percentage averages of *Aspergillus, Pencillium* and *Rhziopus* genera infected were 57, 40, and 3.0 % in almond, 60, 32 and 8 in Pistachio, 53, 33 and 14 % in hazelnut, 50,40 and 10% in Cashew,57, 33 10% in walnuts, 67,24 and 9% in peanut collected from Qassim region , respectively. Infection of these samples with fungi may be due to the bad storage conditions in these region. Results also indicated that, fungi genera were *Aspergillus, Penicillium* and *Rhziopus* in all nut samples. It is known that, food species are common substrates for *A. flavus*, and subsequent aflatoxin production in these food stuffs is almost always due to poor drying, handling, or storage

(Arim, 1995). Jimenez *et al.*, (1991) reported moulds and mycotoxins in almonds, peanuts, hazelnut and pistachio nuts and detected aflatoxins at up to 95 mg/kg in the tested samples. The predominant fungi present in the samples were *A. flavus, A. niger, A. glaucus* Link ex Grey and *Penicillium* spp. In this concern, Vaamonde *et al*., (2003) reported that, the incidence of aflatoxigenic *A. flavus* strains was higher in peanuts (69%) than in wheat (13%) or soybeans (5%) while the ratio of CPA producers *A. flavus* isolated from all substrates was very high (94% in peanuts, 93% in wheat and 73% in soybeans). Isolates of *A. flavus* able to produce simultaneously aflatoxins type B and CPA were detected in all substrates, suggesting the possibility of co-occurrence of these toxins. While, Mphande *et al*., (2004) found that, the account of *Aspergillus* 41% in all the isolates and 98% in peanut samples. the account of *Aspergillus spp*. was 35% of all the isolates, while *Aspergillus niger* being the most prevalent (20.4%). *Aspergillus flavus / parasiticus* were also present and accounted for 8.5% of all the isolates, with *A. flavus* accounting for the majority of the *A. flavus /parasiticus* identified. Regional differences in aflatoxin contamination of crops may be attributable to climatic conditions and to agricultural practices that increase susceptibility of plants to invasion by *A. flavus* (Pildaina *et al.,* 2004).

Occurrence of mycotoxic fungi isolated from nuts :

Mycotoxins are secondary metabolites produced by specific filamentous fungi that contaminate agricultural commodities. Myctoxins produced by an active growing mold. Molds can grow actively without mycotoxin formation. Therefore, these study was carried out to isolate mycotoxic fungi namely *A. flavus* producing aflatoxins.

From Table (2) it could be indicated that, *A. flavus* was the most myctoxins- producing *Aspergillus spp* , represented, 88,78, 84, 46, 80 and 93% of *Aspergillus spp* isolates from almonds, pistachio , hazelnuts, cashews, walnuts, and peanuts collected from Qassim region, respectively were able to produce the aflatoxins.

Results of Table (2) showed that, averages the range of total af latoxins 20-40,12-60,16-50,1.2-6,30-70 and 65-150 mg /L of the yeast extract sucrose (YES) medium were produced by *A. flavus* isolates almonds, pistachio , hazelnuts, cashew, walnuts and peanuts samples collected from Qassim region , respectively. It is worthy to mention that, the current investigation to detect the aflatoxins (B_1, B_2 G_1 and G_2) levels and /or frequencies in the nut. Furthermore, Mphande *et al*., (2004) investigated 32 isolatesof *A. flavus* for their ability of mycotoxin production. They found that, 11isolates did not produce detectable aflatoxins, 8 isolates produced only aflatoxins B_1 and B_2, and 13 produced all four aflatoxins (B_1, B_2, G_1 and G_2) in varying amounts. When the raw peanut samples (n = 120) were analyzed for total aflatoxins, 78% contained aflatoxins at concentrations ranging from 12 to 329 µg/kg. A review of monitoring studies on the occurrence of aflatoxins in food products has demonstrated that aflatoxins are still being found frequently in food products at levels that are of significant concern for consumer protection (Scott and Lawrence, 1997 and Stroka and Anklam, 2002). The occurrence of aflatoxins in dried fruits and nuts was surveyed in the study by Luttfullah and Hussain, (2011) in Pakstan. They found the percentage of contamination for total aflatoxins in the samples such as in; dried apricot (20%), dates (10%), dried figs (50%), dried mulberries (26%) and raisins (20%), while in apricot kernels (26%), almonds without shell (30%), walnuts with shell (40%), walnuts without shell (70%), peanut with shell (40%),peanuts without shell (50%), pistachios with shell (20%), pistachios without shell (50%) and pine nuts with shell (20%).The highest contamination levels of aflatoxins were found in one peanut sample (14.5 mg/kg) and one pistachio sample (14 mg/kg). Molds of the genus *Aspergillus* frequently decay the kernel of pistachio nuts (Mojtahedi *et al.,* 1979). On the other hand, pistachio nuts are among the commodities with the highest risk of AF contamination (Pittet, 1998).

The obtained results of Table (3) and Figures (1,2 and 3) indicated that 80, 80, 60, 40, 40, 20 % for pistachio, peanuts, walnuts, almonds, hazelnuts and cashews samples collected from Qassim region , respectively were contaminated with aflatoxins B_1 and B_2 , aflatoxins G_1 and G_2 .The highly percentage of aflatoxins was found in both Pistachio and peanuts 80% with a concentrations of aflatoxins (AFB_1, AFB_2, AFG_1 and AF G_2) ranged from (11-90, 2-70, 1.1-55 and 1.9-25 "µg/kg") and (70-140,18-71,2.2-10.1and 3.1-13.4 " µg/kg" respectively). Figures 1, 2 and 3 shows the HPLC chromatogram of aflatoxins (G_1,B_1,G_2 and B_2) separation of standards and both positive and negative nuts sample , respectively.

Mycotoxin contamination in some edible dry fruits and nuts has been reported by Zohri and Abdel-Gawad (1993), Abdel-Hafez and Saber, (1993) and Singh *et al*., (2001). Aflatoxins were detected in 90% of hazelnut samples (25–175 mg/kg) and 75% of walnut samples (15–25 mg/kg).In a survey of peanut products in North America, 19% of 1416 samples examined were contaminated with an average level of 1 µg/kg (Stoloff 1977),In Thailand 49% of 216 samples contained AFB1 at an average level of 424 µg/kg (Shank et al. 1972).Aflatoxins are present in food chain consumption of aflatoxin in many parts of the world varies from 0 to 30 000 ng/kg/day (Denning, 1987).Some factors such as high temperature and low moisture can result in cracks in the seed and subsequent invasion by the fungus. Temperature and moisture are the dominant factors that affect aflatoxin contamination of corn. Environmental conditions most favorable for maximum growth and aflatoxin production by *A. flavus* are temperatures greater than 30ºC, maximum relative humidity of greater than 85%, and water activity of 0.98 to 0.99 (Payne *et al.,* 1988). Thus, *A flavus* can infect with proper moisture/temperature conditions during storage almost any stored product (Payne, 1992). Aflatoxin formation in groundnut is favored by prolonged end of season drought and associated elevated temperature (Rachaputi, *et al*., 2002). Singh and

Shukla(2008) investigated fungal infection and mycotoxin contamination in fresh and stored kernels of walnuts collected from different localities of Uttaranchal (India) . They found the species of *Alternaria*, *Aspergillus* and *Penicillium* were predominant. Also, they found that, Thirty-nine percent of *Aspergillus flavus* isolates were toxigenic and produced up to 2170 mg/l of aflatoxin B_1 in the liquid media. Aflatoxin B_1 was the most common mycotoxin encountered as a natural contaminant in the stored samples.

The current investigation was carried out to detect and determine the aflatoxins quantitatively (AFB_1, AFB_2, AFG_1 and AFG_2) and contamination levels in the nuts samples collected from retails markets in Qassim , region .

Conclusion

Studies were carried out to investigate the mycoflora profile and aflatoxin contamination in red nut kernels (almonds, peanuts, hazelnuts, pistachio nuts, walnut and cashew). *Aspergillus flavus, A. niger* and *Penicillium sp* the predominant fungi. The concentration of aflatoxin B_1 was high in peanut and walnut kernels as well as in the toxigenic strains of *A. flavus* isolated from these kernels. There is an urgent need to check the use of contaminated kernels and their products for edible purposes, aflatoxins are potent carcinogens which pose the risk of mycotoxicoses for the consumers.

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