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ORIGINAL ARTICLES

Co-occurrence of fungi, aflatoxins, ochratoxins A and fumonsins in date palm fruits of Saudi Arabia

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ABSTRACT

A survey was carried out to obtain data on the occurrence of most popular mycotoxins (aflatoxins, ochratoxin-A and fumonisins) from date palm in region of Riyadh in Saudi Arabia. The samples were analyzed for alfatoxins by immune affinity column (IAC) clean-up with liquid chromatography and fluorescence detection. 100% of samples showed variable incidence of fungal contamination. The predominant fungi present in date palm samples were *Penicillium* spp., *Aspergillus niger*, and *Rhizopus spp*. The results showed that *Fusarium spp*. was detected only in five varieties (Khalas, Roshodiah, Khadry, Meinifi and Helwa) with frequency occurrence between 2 and 20% and the fungus *F. salani* was the most species isolation. The results also indicated that the highest concentration for aflatoxins total was 5.92ng/g found in Sulaj variety and the aflatoxin B_1 was 5.1ng/g. On the other hand, the Degelat Noor and Dekhaini varieties appeared free from aflatoxins contamination. The highest concentration of ochratoxin-A and fumonisin B_1 was 1.9 ng/gm and 184.5ng/g found in Meneifi and Hulwa variety respectively.

Key words: Mycotoxins, Aflatoxin, Ochratoxin-A, Fumonisin, *Aspergillus*, *Fusarium*, *Penicillium* spp, Date palm

Introduction

Date Palm (*Phoenix dactylifera* L.) is one of the oldest fruit trees in the Arabian Peninsula (AP) and played a key role in the life of its people. Date fruit is marketed all over the world as a high value confectionery and as a fresh fruit it remains an important subsistence crop in most of the desert areas. It is produced largely in the hot arid regions of the world. Fungi are ubiquitous and regarded more or less a source of contamination of foods leading to spoilage and consequently food–borne mycotoxins. So, the advanced countries considered mold and yeast counts as a standard test for checking general sanitary conditions (Foster *et. al*., 1958). Mold growth on foods that are consumed directly can result in direct exposure to mycotoxins (Kurtzman *et al*., 1987).

Mycotoxins mainly produced by certain filamentous fungi belonging to *Aspergillus*, *Penicillium* and *Fusarium* genera. Aflatoxins, ochratoxins, trichothecenes, zearelenone, fumonisins, tremorgenic toxins, and ergot alkaloids are the mycotoxins of greatest agro-economic importance. The Food and Agricultural Organization of the United Nations (FAO) has estimated that up to 25% of the world's food crops are significantly contaminated with mycotoxins (WHO 1999). The economic damage caused by mycotoxins reaches several billion of dollars every year (Khomutov *et al*., 2011). Mycotoxins are highly toxic and cause sever intoxications in human and animals, some of them are carcinogens (Khomutov *et al*., 2011) .

Aflatoxins are group of mycotoxins produced by different species of the genus *Aspergillus*. Naturally occurred aflatoxins include AFB_1, AFB_2, AFG_1, and AFG_2. The potential hazards of aflatoxins to human health have led to worldwide monitoring programs of the toxin in various commodities as well as regulatory actions by almost countries around the world. Considering the extremely high carcinogenicity of aflatoxins, most developed nations regulate limits of aflatoxins as low as reasonably achievable. Aflatoxins may contaminate many crops including peanuts, corn, cottonseed oil, Brazil nuts, pistachios, spices, copra (dried coconut) and figs with widespread contamination in hot and humid regions of the world (Ozer *et al*., 2012) . Several investigators have been surveyed the fungi present in different date palm varieties and studies its susceptibility to produce aflatoxins (Ahmed *et al*., 1997; Ragab *et al*., 2001; Moor *et al*., 2002; Atia *et al*., 2009 and Al-Jasser, 2010).

Ochratoxin-A (OTA) is a secondary metabolite produced by *Aspergillus* and *Penecillium* fungi contaminated a wide variety of foodstuffs. OTA has been proven to have a variety of toxic effects, including nephrotoxic-having been linked to the Balkan Endemic Nephropathy (Vrabcheva *et al*., 2004), immunotoxic

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(Harvey *et al.,* 1992, Abdel-Wahhab *et al.,* 2005), carcinogenic, teratogenic (Abdel-Wahhab *et al*., 1999; Wangikar *et al*., 2005), while also being a possible genotoxic (Abdel-Wahhab *et al*., 2008). OTA has been classified as a possible human carcinogen (category 2B) by the International Agency for Research on Cancer (IARC, 1993) and having been linked to the Balkan Endemic Nephropathy (Vrabcheva *et al*., 2004 and Milicevic *et al*., 2008). This mycotoxin occurs in various foodstuffs and beverages including a variety of cereals, beans, coffee, instant coffee, beer, wine, meat, cocoa, dried fruits, spices, nuts, milk, pig blood and kidney and other tissues of animal origin (Wilson *et al*., 2002, CAST, 2003 and Soubra *et al.,* 2009).

Fumonisins are a family mycotoxin produced by *Fusarium verticillioides* (Dutton, 1996). It is well known that fumonisins induce several animal diseases, including leukoencephalomalacia in horses (Marasas, *et al.*, 1988), and cancer in rats (Gelderblom *et al.,* 1991). Furthermore, in some areas of China and South Africa, the consumption of fumonisin-contaminated maize is correlated with high incidences of human esophageal cancer (Sydenham *et al.*, 1990 and Menniti and Neri, 2010).

The present study was conducted to determine the fungal species contamination in date palm fruits and to detect the possibility of mycotoxin production due to the occurrence of these fungi under natural conditions in Riyadh City, Saudi Arabia.

Material and Methods

Sample collections:

Twelve samples of semi-dry dates namely Sukkari, Dokeiny, Salg, Razeiz, Sakiee, Khalas, Roshodiah, Nabtat Aly, Khattary, Meinifi, Dooglet, and Helwa were collected from different markets of Riyadh in Saudi Arabia for aflatoxins, ochratoxin A, fumonisins toxins and fungal count analysis. Samples were directly placed in a sterile polyethylene bag which was subsequently sealed. The bags were transferred to the mycological lab and kept in a refrigerator at 4-5 ºC.

Isolation and identification of fungi:

Isolation of the accompanied fungi was done using the culture plate technique adopted by Johnson and Curl (1972) using two media, potato dextrose agar (PDA) containing 20 µg/ml chloramphenicol and 30 ppm rose bengal and malt agar media. Ten grams of each sample were aseptically removed and transferred to sterile blender jars. The content was homogenized before serially up to 10^4. Aliquots of the serial dilutions were surface plated in duplicate on the two media, then incubated for 7 days at 28±2ºC. Incubated dishes were daily examined and developing fungi were counted, in terms of cfu/g then sub-cultured and purified on PDA medium.

The obtained fungi were identified to the generic or species based on culturable and morphological characteristics using the identification key's and according to using the description of Booth (1971), Nilson *et al*.,(1983) and Barnett and Hunter,(1986).

Mycotoxins analysis:

Apparatus:

The High Performance Liquid Chromatography (HPLC) system consisted of Waters Binary pump Model 1525, a Model Waters 1500 Rheodyne manual injector, a Waters 2475 Multi-Wavelength Fluorescence Detector, and a data workstation with software Breeze 2. A phenomenex C_{18} (250x 4.6 mm i.d), 5 um for aflatoxins**.** A Symmetry C_{18} (5 µm particle size, 150 mm X 4.6 mm i.d.) from Waters corporation (USA) for ochratoxin A. A Hyper Clone 5 µ ODS column (C_{18}) 120A° , DIM: 250 x 4.60 mm. (Phenomenex) for fumonisins.

Extraction of aflatoxins by VICAM method:

Twenty five grams of sample with 5 g sodium chloride place in blender jar with 125 ml methanol: water (70:30). Cover blender jar and blend at high speed for 1 minute. Remove cover from jar and pour extract into fluted filter paper. Collect filtrate in a clean vessel and

15 ml of filter extract was transferred to a clean vessel. Dilute extract with 30 ml of purified water. Mix well the filter dilute extract through glass microfiber filter into a glass syringe barrel using markings on barrel to measure 4 ml.

Immunoaffinity Chromatography:

15 ml filtered diluted extract (15 ml equivalent 1g sample) passed completely through AflaTest ®-P (VICAM, Watertown, mA 02472 USA) affinity column at a rate of about 1-2 drops/second until air comes through column. Pass 5 ml of purified water through the column at a rate of about 2 drops/second. Elute affinity column by passing 1.0 ml HPLC grade methanol through column at a rate of 1-2 drops/second and collecting all of the sample eluate (1ml) in a glass vial. Evaporated to dryness under stream of nitrogen and was determination of HPLC.

Determination of Aflatoxins by HPLC:

Derivatization:

The derivatives of samples and standard were done according to (AOAC, 2007). The mobile phase consists of acetonitile/water/ methanol (1:6:3). The separation was performed at ambient temperature at a flow rate of 1.0 ml/min. The injection volume was 20 µL for both standard solutions and sample extracts. The fluorescence detector was operated at an excitation wavelength of 365 nm and an emission wavelength of 450 nm. AFB_1 concentration in samples was determined from the standard curve, using peak area for quantitation.

Extraction of ochratoxin A by VICAM method:

Methods developed by the immunoaffinity column provider (VICAM, Ochratest; Watertown, mA 02472 USA) was utilized for sample preparation, extraction and for the RP-HPLC conditions used for OTA. Twenty grams of sample was mixed with 100 ml of pure water and homogenized with a blender at high speed for 3 min to obtain a homogeneous sample. 100 ml of 2% sodium bicarbonate was added to the slurry and blended at high speed for 2 min to extract the OTA from the sample. Then, the mixture was filtered though filter paper collected in a beaker (10 ml) and diluted with 10 ml of phosphate buffered saline (PBS) solution. This diluted solution was passed through an immunoaffinity column (VICAM, Ochratest: Watertown MA 02472 USA). OTA was eluted from the column by addition of 1.5 ml HPLC grade acidified methanol (aceticacid:methanol 2:98) and then 1.5 ml of HPLC grade water and gravity was used to collect the eluate into a glass vial.

Detection and Determination of Fumonisins:

The fumnosins analysis was carried out according to (AOAC, 2007).

Extraction and cleanup of Fumonisins:

FBs were extracted by a modification of the procedure of (Jimenze and Mateo, 1997). Briefly, Fifty grams was blended in Waring Blender with 100 ml methanol: water (3:1; v/v) for 5 min. A 10 ml aliquot was diluted with 40 ml of PBS pH 7.0 mixture. 10 ml of diluted extract was passed through Fumoni Test affinity column. After that pass 10 ml of PBS through the column at a rate 1-2 drops/second until air comes through the column. The fumonisin was eluted from the column by passing 1.5 ml methanol HPLC in glass vial. The sample was evaporated to dryness under N2 at 40°C and derivatized according to the method of (AOAC, 2007).

Results and Discussion

Fungal flora associated date palm fruits:

Table (1) show the total fungal count (cfu/g) of the 12 most popular date palm fruits received at Tamr stage of maturation. Data recorded indicated that the total viable count was ranged from 2 to 250 cfu/g on PDA medium and from 0 to 500 cfu/g on malt extract agar medium. Data also showed that levels of isolated fungi differed among the different varieties. Khalas date palm variety has higher load of contaminated fungi (375 cfu/g) followed by Salg variety (181cfu/g), Roshdiah (110 cfu/g) and Helwa (90 cfu/g) with an average percentage occurrence of 35.3%, 17.05%, 10.36% and 8.48% respectively. One the other hand, Nabtat Aly, Sukkari and Raziez varieties had the lower load (1.11 and 18.5 cfu/g respectively) with an percentage occurrence of 0.09%, 1.04%, 1.045 and 1074% respectively.

Table 1: Total fungal count (cfu x 10) and percentage occurrence of fungi associated with date palm seeds isolated on PDA and malt media for 7days at 28±2ºC.

Varieties	Media used		Mean	Percentage occurrence
	PDA	Malt		
Sukkari	2	20	11	1.04
Dekhaini	100	20	60	5.65
Salg	200	162	181	17.05
Razeiz	7	30	18.5	1.74
Sakiee	40	20	30	2.83
Khalas	250	500	375	35.33
Roshodiah	200	20	110	10.36
Nabtat Aly	2	0	1	0.09
Khattary	110	90	100	9.43
Meinifi	100	10	55	5.18
Dooglet	30	30	30	2.83
Helwa	80	100	90	8.48
Total	1121	1002	1061.5	

Generally, there was a higher incidence of diverse fungi contaminated in the samples. These results were agreed with those obtained by Atia *et al* (2009) and Al-Jasser (2010), On the other hand, these results were in contrast with those obtained by Abu-Zinada and Ali (1977), Nassar (1986), Abdel-Satar and Saber (1999). They reported that the dry dates were highly polluted with various fungal genera and species. Similar to these results in another study in Egypt, Abdel-Satar and Saber (1999) found that *Aspergillus* was the most frequently isolated genus with contamination of 100% of the date palm, while *Penicillium* was less frequently isolated with contamination of 30% of the date samples.

Concerning the fungal taxa, forty species belong to six genera were isolated and identified from the collected samples tested (Table, 2). Members of *Aspergillus, Penicillium and Rhizopus* were the most prevalent.

Table 2: Total fungal count(cfu/g) and frequency occurrence percentage of fungi associated with date palm seeds isolated on PDA medium for 7days at 28±2ºC

Varieties	*Alternaria alternata*	*Aspergillus candidus*	*A. flavus*	*A.niger*	*A. sydowi*	*A. terreus*	*A. versicolor*	*Fusarium solani*	*F. oxysporum*	*Mucor spp*	*Penicillium chrysogenum*	*P. frequentans*	*Rhizopus nigricans*	*R. oryzea*
Sukkari	-	-	-	*100 **(2)	-	-	-	-	-	-	-	-	-	-
Dekhaini	-	-	4 (4)	90 (90)	-	-	-	-	-	-	-	-	5 (5)	1 (1)
Salg	-	-	-	75 (150)	-	-	12.5 (25)	-	-	-	-	-	12.5 (25)	-
Razeiz	-	-	14.3 (1)	42.9 (3)	-	-	-	-	-	-	14.3 (1)	-	28.6 (2)	-
Sakiee	2.25 (1)	-	-	75 (30)	7.5 (3)	-	12.5 (5)	-	-	-	2.5 (1)	-	-	-
Khalas	20.0 (50)	2 (5)	10 (25)	12 (30)		2 (5)	-	2 (5)		20 (50)	10 (25)		20 (50)	2 (5)
Roshodiah	-	-	-	50 (100)	-	12.5 (25)	-	-	12.5 (25)	-	12.5 (25)	-	12.5 (25)	-
Nabtat Aly	-	-	-	50 (1)	-	-	-	-	-	-	-	-	50 (1)	-
Khattary	-	-	-	81.8 (90)	-	-	-	4.6 (5)	-	4.6 (5)	4.6 (5)	-	1.8 (2)	2.7 (3)
Meinifi	-	10 (10)	-	10 (10)	-	10 (10)	-	20 (20)	-	10 (10)	10 (10)	10 (10)	10 (10)	10 (10)
Deglet Noor	16.7 (5)	-	16.7 (5)	33.3 (10)	-	-	-	16.7 (5)	-	-	10 (3)	-	16.7 (5)	-
Hulwa	-	-	-	50 (40)	-	-	12.5 (10)	-	-	12.5 (10)	12.5 (10)	-	12.5 (10)	-

*= frequency occurrence percentage
**= Total fungal count(cfu/g)

Aspergillus was the first predominant genus encountered in 3.0 to 100% of the total fungi. Six *Aspergillus* species (*A. candidus, A. flavus, A. niger, A. sydowi, A. terreus and A. versicolour*) were identified. *A. niger* was the most detected species in all samples of date palm varieties (2 to 150cfu/g) with frequency occurrence of 75 to 100%, followed by *A. flavus* (1to 25cfu/g) with frequency occurrence of 4.0 to 25%. Whereas, *A. versicolour* was isolated from three date palm varieties with frequency occurrence of 12.5% and *A. terreus* between (2 to

25%). The remaining *A. candidus* was encountered only from two varieties (khalas and Meinifi) with frequency occurrence between 2 to 10%. These results are generally in agreement with those obtained by Nassar (1986), Abdel-Satar and Saber (1999), Ragab *et al*. (2001). They found that *Aspergillus* spp. was the predominant genus on dry date palm fruits and the most prevalent species were *A. niger* and *A. flavus*.

On the same line, Hasnaoui *et al.* (2010) reported that the most abundant genus found in date palm fruits collected from morocco was *A. niger*, while, Al-Sheikh (2009) isolated 16 genera of fungi from local varieties grown in Saudi Arabia including, *A. niger* and *A. flavus*.

The second higher incidence rate was represented by the genus *Rhizopus*. It recovered from ten date palm varieties with frequency occurrence between 1 to 50%. Among the two isolated *Rhizopus spp*., *R. nigricans* was the most common, whereas *R. orizae* was isolated only from four date palm varieties with frequency occurrence between 1 to 10%. This genus was also isolated from dry and semi dry dates, as reported by Bokhary (2010).

The third higher incidence rate represented by the genus *Penicillium*. It recovered from fruits of eight date palm varieties with frequency occurrence between 25 to 14.3 %. Two species of *Penicillium*, i.e., *P. chrysogenum* and *P. frequentans* were identified and the later fungus was found to infect only date fruits of Meinifi variety at 10cfu/g. *Fusarium spp*. was detected only in five varieties (Khalas, Roshodiah, Khadry, Meinifi and Helwa) with frequency occurrence between 2 and 20% and the fungus *F. salani* was the most species isolation, especially on Meinifi variety.

Data in Table (2) also showed that *Alternaria alternate* was detected as a single representative of genera with frequency between 2.5 to 20% on three date palm fruits of Sakiee, Khalas and Deoglet varieties. These results are inagreement with Ibrahim and Rahma (2009), Al-Sheikh (2009) and Bokhary (2010).

Natural occurrence of some mycotoxins in date palm fruits:

1-Aflatoxins:

The results in Table (3) showed the natural occurrence of aflatoxins in different varieties of date palm fruits. The results indicated that the concentrations of aflatoxins were ranged from 0.00 to 5.92 ng/g sample.

Table 3: Natural occurrence of aflatoxin, ochratoxin A and fumnosins in dried dates.

Cultivars	Aflatoxins concentration ng/g					Ochratoxin A ng/g	Fumonisins ng/g	
	G1	B1	G2	B2	Total		B1	B2
Sukkari	0.22	2.4	ND	1.6	4.22	0.8	70	33
Dekhaini	ND	ND	ND	ND	ND	0.7	20	ND
Sullaj	0.22	5.1	ND	0.6	5.92	1	111.3	20.6
Ruzeiz	0.42	1.8	ND	0.6	2.82	0.5	ND	ND
Sakki	0.21	0.83	ND	0.6	1.64	0.6	25.8	6.8
Khalas	0.21	1.72	ND	0.6	2.53	1.2	ND	ND
Rushodia	0.21	1.1	ND	0.58	1.89	1.3	ND	ND
Nabtat Ali	ND	1.8	0.52	ND	2.32	1.4	ND	ND
Khodry	0.23	0.51	ND	ND	0.74	0.8	ND	ND
Meneifi	0.30	1.40	ND	ND	1.70	1.9	ND	ND
Deglet Noor	ND	ND	ND	ND	ND	1.4	73.8	ND
Hulwa	0.30	ND	ND	ND	0.30	0.4	184.5	ND

The results also indicated that the highest concentration for aflatoxins total was 5.92 ng/g found in Sulaj vareity and the aflatoxin B_1 was 5.1ng/g. On the other hand, the Degelat Noor and Dekhaini varieties appeared that free from aflatoxins contamination. In the similar study carried out by Ragab *et al* (2001) survived 50 date palm samples with different processing. The results show that most of the samples to be aflatoxins free expect 2 samples out of 5 samples of pitted fruits stuffed with peanut which contained aflatoxin. The aflatoxin B_1 concentration detect in the two contaminated samples were 4.8 and 6.2 ng/g. In previous study carried out by Abdel-Sater and Saber (1999) reported that aflatoxin B_1 was found in 2 samples out of 20 tested of dry date. Limited reports have been published about the occurrence of aflatoxins in date palm.

In this concern, the date fruit may be are less suspected contaminated with aflatoxins because the chemical composition of the date fruit. Since, aflatoxins are produced under certain conditions that include temperature 13–40°C (optimum 30 °C) and a_w of 0.95 (Giorni *et al.,* 2009). Moreover, AFB_1, B_2, G_1 and G_2 are generally found in fat containing food and feed like ground nuts and their processed products (Abdel-Wahab *et al.,* 2011).

2- Ochratoxin-A:

Results in Table (3) showed that out of 12 date palm samples analyzed, 11 samples (91.1 %) were contaminated with ochratoxin-A. Data also showed that ochratoxin-A in contaminated samples ranged from 0.4-1.9 ng/g. This result is line with that results obtained from the date analysis with total fungus counts. The results

showed that the tolerable level of ochratoxin-A, which are carried out by European regulation were less in date samples.

Several investigators reported that humans are exposed to ochratoxin-A via risky foods such as cacao, coffee, grapevine, dried fruits and various spices (Brera *et al.,* 2002 and Colak, 2006). In the fact that date is on high demand in the Arabian countries, only very limited published studies about occurrence of OTA are found in the literature.

3- Fumonisin:

Results in Table (3) show the occurrence of FB_1 and FB_2 in the 12 dried dates samples collected from the market in Saudi Arabia. FB_1 contamination has been determined between 0.00 -184.5 ng/g and FB_2 between 0.0 - 33 ng/g. There are no legal regulations regarding FBs levels in dried date or fruits. Maximum levels of FBs for unprocessed maize (2µg/g) and for maize flour, maize grits and maize meal (1µg/g) have been set by the European Union (EC, 2006). In this study the FBs level is lower than the unprocessed maize regulation.

Conclusion:

Despite the presence of fungal contamination secreting mycotoxins and also aflatoxins, ochratoxin-A and fumonsin mycotoxins found, it in the allowable limits and borders safe for human consumption.

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