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Mycobiota and Incidence of Toxigenic Fungi in Dried Fruits from Duhok Markets, North Iraq

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ABSTRACT

Thirty samples from each of four dried fruits (apricot,fig,plum and raisins) collected from local shops at Duhok governorate were surveyed for their contamination with fungi. Thirty eight fungal species belonged to 13 genera in addition to yeasts were isolated and identified. The highest diversity of fungi were detected from raisins (35 species), followed by 27 species isolated from plum, 26 species from figs and 18 species on apricot. Eleven species were found common on the four types of dried fruits. These include *Alternaria alternata, Aspergillus carbonarius, A. flavus, A. fumigatus, A. niger, A. parasiticus, Penicillium citrinum, P. expansum, Cladosporium cladosporoides, Emericella nidulans and Eurotium amstelodami*. Aflatoxigenic potentials of selected isolates of *Aspergillus* section *Flavi* and ochratoxigenic potential of selected isolates from *Aspergillus* section *Nigri* were detected by ELISA technique. Aflatoxin was found at levels from 79.4 to 356ppb whereas, ochratoxin A at levels from 60-106ppb.

INTRODUCTION

Dried fruits are susceptible to fungal contamination and mycotoxin production because of their favorable moisture, high level of sugar content and other nutrients (Embaby *et al.* 2012). Fungal infection to dried fruits may occur on the tree during ripening stages, after falling from the tree and during drying process (Ozay *et al.*1995; Heperkan *et al.* 2012a).

Mycotoxins are secondary metabolites synthesized by several filamentous fungi that grow on various agricultural products and foodstuff (Kumar *et al.*2008). The most important genera of filamentous fungi that grow and produce mycotoxins in food and dried fruits are *Aspergillus, Fusarium* and *Penicillium* (Pitt *et al.*, 2000; Ozer *et al.* 2012). Although a large number of mycotoxins exist, two of them, namely aflatoxins (AFs) and ochratoxin A (OTA) are frequently detected from dried fruits (Zohri and Abdel-Gawad, 1993; Trucksess and Scott, 2008; Ozer *et al.* 2012).

Aflatoxins are produced by members of *Aspergillus* section *Flavi*. *A. flavus* and *A. parasiticus* have been considered the most important aflatoxin producers (Pitt and Hocking, 2009). However, few species of *Aspergillus* in section *Circumdati* and in section *Nidulans* have been also found to produce aflatoxin (Cary *et al*. 2005). Aflatoxins are potent hepatotoxic and carcinogenic toxin causing health hazards to human and animals (Hedayati *et al*. 2007)

Ochratoxin A was first isolated from *A. ochraceus* from South Africa in 1965 (Van der Merwe *et al*. 1965). Subsequent investigations revealed that OTA is produced by several *Aspergillus* species belonging to sections *Circumdati, Flavi* and *Nigri* (Frisvad *et al*. 2004; Samson *et al*. 2004). Among *Penicillium* species, Larsen *et al*. (2001) reported *P. verrucosum* and *P. nordicum*, whereas, Vega *et al*. (2006) reported *P. brevicompactum, P. crustosum, P. olsoni* and *P. oxalicum* as OTA producers. OTA is a potent nephrotoxic mycotoxin that has been linked to kidney problems in both livestock and human populations (Petzinger and Ziegler, 2002).

The aim of this work was to survey the fungi contaminated four types of dried fruits and to asses *in vitro* the aflatoxin and ochratoxin A producing potential of some fungal isolates using ELISA technique.

MATERIALS AND METHODS

Dried fruit samples

Dried fruit samples (30 samples each from apricot, figs, plum and raisins) were collected randomly from local markets in Duhok governorate. The collected samples were put in paper bags and were brought into laboratory for fungal isolation.

Mycological analyses

Larger dried fruits (apricot, figs and plum) were cut aseptically into small pieces, whereas, fruits (raisins) were analyzed as whole piece. The fruit pieces were surface disinfected with 2% sodium hypochlorite for 1 min., and then rinsed with sterile distilled water. Ten pieces were placed onto Dichloran Rose Bengal Chloramphenicol (DRBC) agar medium (Fluka-Germany) and examined daily for growth and sporulation of fungi for 7 days using a stereomicroscope.

Identification of fungi

Pure colonies were established on appropriate media for identification. Majority of detected species were identified to species level based on morphological and cultural characteristics. Fungi other than the genera *Aspergillus* and *Penicillium* were identified according to the manuals of Domsch *et al.*,(1980) and Pitt and Hocking, (2009).

For identification of species in the genera *Aspergillus* and *Penicillium*, pure colonies were grown on four media according to Klich (2002) and Samson *et al.*, (2000). The media are as follows: Czapeck Yeast Extract Agar incubated for seven days at 25C˚ (CYA25), Czapeck Yeast Extract Agar incubated for seven days at 37C˚ (CYA37), Czapeck Yeast Extract Agar with 20% Sucrose incubated for seven days at 25C˚ (CY20S), Malt Extract Agar (MEA) incubated for seven days at 25C˚.

Ingredients and preparation of the above five media were mentioned in Klich (2002), Pitt and Hocking (2009). Each medium was supplemented with 250mg / L chlorophenicol (SDI) to suppress bacterial growth. For each culture four plates were used, two of CYA and one each of CY20S, MEA. Each plate was inoculated at the center and incubated in the dark for seven days. One CYA was incubated at 37C˚. The rest were incubated at 25C˚. All species identifications were according to the keys and descriptions provided by Klich (2002); Samson *et al.*, (2004); Frisvad *et al.*, (2004); Samson *et al.*, (2007); and Noonim *et al.*, (2008); Pitt and Hocking (2009).

Isolation frequency of fungal species from samples was calculated by applying the following formula.

$$\text{Isolation frequency \%} = \frac{\text{Number of samples on which a fungus appeared X 100}}{\text{Total number of tested samples}}$$

Aflatoxin extraction from fungal cultures

Production of aflatoxin (AF) by randomly chosen isolates of *Aspergillus*

section *Flavi* was screened according to the method of Bragulat *et al.*, (2001) by centrally inoculating yeast extract sucrose (YES) plates and then incubated in the dark at 25^{o} C for 7 days. Agar plug (0.5 cm) diameter was removed from the edges of the centre of the colony and a midway between the edge and the centre of the growing colonies. The three plugs were mixed with 1 ml methanol in a small vial ,shaking vigorously and left at room temperature for 1h, mixed again and the extracts were filtered through milpoore filter (0.22 um) diameter (Millex GP Filter Unit Coringhwohill Co. Ireland).

Ochratoxin A extraction from fungal cultures

Isolates from *Aspergillus* and *Penicillium* genera were evaluated for their ochratoxin A producing potential.The method of Bragulat *et al*. (2001) for extraxtion from fungal cultures was adopted. The extracts were filtered through milpoore filter (0.22 um) diameter (Millex GP Filter Unit Coringhwohill Co. Ireland).

Aflatoxin and ochratoxin A analysis

The quantitative analysis of AF was performed with the enzyme linked immunosorbent assay (ELISA). The aflatoxin assay was performed according to the instructions provided by the manufacture (Veratox Aflatoxin quantitative test, Neogen Corporation, USA). Aflatoxin produced by isolates was calculated from the standard curve derived from aflatoxin standards and expressed in ppb.OTA assay was performed according to instructions provided by the manufacture (Veratox quantitative ochratoxin test, Neogen Corporation USA). Ochratoxin produced by isolates was calculated from the standard curve derived from ochratoxin standards and expressed in ppb.

RESULTS AND DISCUSSION

The fungi contaminated four types of dried fruit samples collected from Duhok shops and their isolation frequencies were presented in Table 1. Thirty eight fungal species represented 13 genera in addition to yeasts were identified. The highest diversity of fungi were detected from raisins (35 species), followed by 27 species isolated from plum, 26 species from figs and 18 species on apricot. The majority of the recovered species were previously reported from dried fruits in many parts of the world (Zohri and Abdel-Jawad, 1993; Alghalibi and Shater, 2004; Iamnaka *et al*. 2005, 2007; Ozer *et al.* 2012; Sen and Nas, 2013).

Aspergillus was represented by 15 species and thus showed the widest diversity among all recovered genera. Black aspergilli (*Aspergillus* section *Nigri*) were represented by 6 species. These include *A. aculeatinus. A. aculeatus, A. brasiliensis, A. carbonarius, A. japonicus* and *A. niger*. These species were frequently isolated from soil and from different agricultural commodities in Duhok, north Iraq (Abdullah and Abdullah, 2009; Abdullah and Muhammed, 2011; Saadullah and Abdullah, 2012 a,b,c; Abdullah and Saadullah, 2013).

Eleven species were found common on the four types of dried fruits. These include *Alternaria alternatta, Aspergillus carbonarius, A. flavus, A. fumigatus, A. niger, A. parasiticus, Penicillium citrinum, P. expansum, Cladosporium cladosporoides, Emericella nidulans* and *Eurotium amstelodami*. These results are in line with those obtained by Zohri and Abdel-Jawad (1993); Alghalibi and Shater (2004).

The most frequently isolated species from apricot was *A .niger*, followed by *A. carbonarius* and *A. flavus* with a percentage frequencies 78%, 40% and 36% respectively, whereas; the most frequently isolated species from plum was *A. niger* (66.7%), followed by *A. flavus* (45%) and *A. carbonarius* (30.3%). Iamanaka *et al*. (2005) stated that the most frequently detected species from dried plum in Brazil were ,*A. niger*, followed by *Penicillium* spp. and *A. ochraceus*, whereas; samples from dried apricot were not contaminated by any fungi.

Table 1: Percentage occurrence of fungi on dried fruits as detected on DRBC medium

Fungal species	Isolation frequency (%)			
	Apricot	Figs	Plum	Raisins
Alternaria alternata (Fr.) keissler	7.71	6.0	6.0	4.2
Aspergillus aculeatinus Frisvad, Varga & Samson	3.0	0.0	6.0	7.5
A. aculeatus Lizuka	0.0	10.12	0.0	10.12
A. alliaceus Thom & Church	10.0	13.22	0.0	3.0
A. brasiliensis Varga, Frisvad & Samson	0.0	1.7	3.0	5.0
A. candidus Link	0.0	0.0	3.3	5.0
A. carbonarius (Bainier) Thom	40.0	31.3	30.3	60.3
A. clavatus Desm	0.0	3.0	1.0	3.1
A. flavus Link	36.0	66.0	45.0	31.2
A. fumigatus Fresen	10.0	10.0	8.1	7.3
A. japonicus Saito	0.0	5.50	3.33	5.50
A. niger Tiegh	78.0	73.4	66.7	92.3
A. ochraceus K. Wilh	0.0	10.0	2.0	6.0
A. oryzae (Ahlburg.) Cohn	3.0	0.0	3.0	0.0
A. parasiticus Speare	28.5	31.5	10.0	7.12
A. terreus Thom	0.0	0.0	0.0	2.5
Penicillium brevicompactum Dierckx	13.0	0.0	10.0	13.0
P. citrinum Thom	13.0	18.0	13.0	18.0
P. expansum Link	15.0	7.0	12.0	12.0
P. funiculosum Thom	0.0	0.0	10.0	7.0
P. glabrum (Wehmer)Westling	0.0	11.33	3.0	20.0
p. oxalicum Currie&Thom	1.0	2.66	0.0	2.66
Cladosporium cladosporoides (Fresen.) G. A. de Vries	2.2	3.33	3.1	6.0
C. herborium (Pers.) Link	0.0	1.0	0.0	3.2
Chaetomium sp.	0.0	0.0	0.0	3.0
Emericella nidulans (Eidam) Vuil	7.0	3.33	2.0	1.0
E. quadrileneata Thom & Raper	0.0	3.5	0.0	3.5
E. rugulosa C. R. Benj.	0.0	0.0	0.0	2.5
Eurotium amstelodami L. Mangin	17.0	6.0	3.33	10.3
E. chevarlleri L. Mangin	13.0	0.0	10.2	10.2
E. herborium Link	0.0	0.0	13.0	15.0
Fusarium sp.	0.0	3.0	1.0	0.0
Geotrichum candidum Link ex Fr.	1.0	1.0	0.0	1.0
Gliocladium sp.	0.0	4.0	1.5	0.0
Rhizopus stolonifer (Ehrenb.)Vuill.	0.0	0.0	10.0	1.5
Stachybotrys sp.	0.0	3.33	0.0	3.0
Ulocladium atrum Preuss.	0.0	0.0	0.0	12.0
Yeasts	0.0	4.0	3.0	3.0

The two black aspergilli (*A. niger* and *A. carbonarius*) were the most fungal species isolated from raisins and with percentage frequency of 92.3% and 60.3% respectively. Black aspergilli were also reported as the most common species on raisins in several studies in different countries (Magnoli *et al*. 2004; Hakobyan *et al*.2010; Palumbo *et al*. 2011). The most encountered species from dried figs were *A. niger, A. flavus, A. carbonarius* and *A. parasiticus* with a percentage frequencies of 73.4%, 66.05, 31.3, 31.5% respectively. Embaby *et al*. (2012) recorded *A. niger, A. flavus* and *A. parasiticus* as the most frequent species on dried fig in Egypt. Similar results were reported by Doster *et al*. (1996) from figs in California. However, on Turkish dried figs, *A. flavus* and *A. parasiticus* in general instances and in very rare cases *A. niger* and *A. fumigatus* were detected as the predominant species (Steiner *et al*. 1988). Javanmard (2010) reported that the most frequent species in Iranian dried figs was *A. niger* aggregate (90%) followed by *A. flavus* (63.76%).

Penicillium was second in the number of species isolated from dried fruits and was represented by six species. *P. citrinum* and *P. expansum* were the most common species and were detected from the four types of dried fruits. Zohri and Abdel-Jawad (1993) reported that *Penicillium* was the most predominant genus isolated from dried apricot and prunes in Egypt. The genus was represented by four species of which *P. chrysogenum* was the most common species in the two dried fruits. Senyuva *et al*. (2008) isolated *P. expansum* and *P. chrysogenum* as the most frequent species on Turkish dried figs, whereas; *P. chrysogenum* and *P. expansum* were detected in high frequency on dried figs in Yemen (Alghalibi and Shater, 2004).

The two teleomorphic ascomycetes (*Emericella* and *Eurotium*) were represented each by three species. Among them, *Emericella nidulans* and *Eurotium amstelodami* were the most frequent species and were detected from the four types of dried fruits. *Cladosporium* was represented by two species *viz C. cladosporoides* and *C. herborium*. The rest of genera were represented by one species each.

Table 2 showed the results of screening *Aspergillus* section *Flavi* strains for aflatoxins production abilities and isolates from *Aspergillus* section *Nigri* for Ochratoxin A production potential in culture media as detected by ELISA. Two isolates of *A. flavus* out of four were negative for aflatoxin production, whereas; all tested isolates of *A. parasiticus* showed positive abilities. The tested isolates from both *A. flavus* and *A. parasiticus* showed marked variations in their aflatoxin potential ranging from 79.4 to 334 ppb in *A. flavus* and from 81 to 356ppb in *A. parasiticus*.

Table 2: Quantitative production of aflatoxin and ochratoxin A by *Aspergillus* species *in vitro* by ELISA technique

Fungal species	Source	Aflatoxin ppb	Ochratoxin A ppb
Aspergillus flavus isolate 1	Raisins	79.4	-
A. flavus isolate 2	Raisins	N.D	-
A .flavus isolate 3	Fig	334	-
A. flavus isolate 4	Plum	N.D	-
A. parasiticus isolate 1	Fig	344	-
A. parasiticus isolate 2	Plum	356	-
A. parasiticus isolate 3	Fig	345	-
A. parasiticus isolate 4	Fig	81	-
A. parasiticus isolate 5	Raisin	266	-
A. niger isolate 1	Raisin	-	N.D
A. niger isolate 2	Raisin	-	60
A. carbonarius isolate1	Raisin	-	73
A. carbonarius isolate 2	Apricot	-	106
A. japonicus	Apricot	-	N.D
A. ochraceus	Plum	-	N.D

Abdullah and Al-Mousawy (2009) showed that out of 24and 18 isolates of *A. flavus* obtained from corn grains and sunflower seeds respectively, 15 isolates (62. 5%) from corn and 10 isolates (55.5%) from sunflower seeds showed a positive aflatoxin activity. However, in Iraq, more recently Mohammed *et al.(*2010) showed that 81.8% of *A. flavus* isolates and 100% of *A. parasiticus* isolates were positive. Not all strains of *Aspergillus* section *Flavi* can produce aflatoxin and the ratio of the non-aflatoxigenic strains to aflatoxin producing strains varied according to the source and location of the isolates (Schroeder and Bolla, 1973; Abdel-Malek *et al*.1993).

Out of six isolates of *Aspergillus* section *Nigri* screened for their ochratoxin A potential, one isolate of *A. niger* and two isolates of *A. carbonarius* were positive for

ochratoxin A production. Ochratoxin A produced by *A. niger* isolate 2 and *A. carbonarius* isolate 1 was 60 and 73 ppb respectively, whereas, *A. carbonarius* isolate 2 derived from apricot dried fruit showed the highest potential (106ppb).OTA production by black aspergilli isolated from dried fruits have been reported by several studies (Magnoli *et al*. 2004; Iamanaka *et al*. 2005; Leong *et al*. 2006; Palumbo *et al*. 2011; Heperkan *et al*. 2012b). In all of these studies, the majority of ochratoxigenic isolates were assigned to *A. carbonarius*, while very few isolates of *A. niger* aggregate were produced OTA.

CONCLUSION

The result of this study revealed that dried fruits harbor a diversity of fungal contaminants. Some of these fungi isolated are capable of producing aflatoxins and Ochratoxin A and thus there may be risk through consumption of these dried fruits. Therefore, strict hygienic mycological investigation should be done during harvest, storage and drying to minimize contamination with such fungi.

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