Ass. Univ. Bull. Environ. Res. Vol. 7 No. 2, October 2004

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MYCOFLORA AND MYCOTOXIN CONTAMINATION OF SOME DRIED FRUITS IN YEMEN REPUBLIC

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ABSTRACT :

The mycoflora analysis of some dried fruits in Yemen Republic showed a wide range of fungal contamination in 60 samples collected from different markets in Sana'a, Taiz, Adan and Ibb governorates. Twenty three species and one variety belonging to 15 genera were isolated from dried fruits (raisins, dates and figs) on two types of media. *Aspergillus niger*, *A. flavus, A. fumigatus, A. ochraceus, Penicillium chrysogenum* and *Rhizopus stolonifer* were the most common fungal species isolated on 1% glucose–Czapek's agar medium at 28ºC, while *Eurotium amstelodami, Zygosaccharomyces rouxii, A. niger* and *P. chrysogenum* were common on 40% sucrose-Czapek's agar.

The dried fruit samples were analyzed for the presence of different mycotoxins. Thin Layer chromatographic analysis of chloroform extracts of dried fruits revealed that six samples of dried raisins, two samples of dried figs and two samples of dates were naturally contaminated with aflatoxin B1. The concentrations of aflatoxin were ranged between 130-350 µg/kg of raisins, 120-250 µg/kg of figs and 110-180 µg/kg of dates. Ochratoxin A was detected in four samples of figs (70-160µ/kg). The other mycotoxins under investigation were not detected. Bioassay test using *Artemia salina* larvae showed that chloroform extracts of 14 dried samples were toxic.

INTRODUCTION:

In most developing countries, agriculture is the back bone of the economy and export crops are greatly depended upon as a source of foreign exchange to finance productive activities and other essential services. Most of these crops are cereals, fruits, vegetables and oil seeds that are highly susceptible to fungal growth and mycotoxin production (Garbutt, 1997). Mycotoxins are toxic metabolites of fungi which, if ingested, can cause acute or chronic toxic effects such as carcinogenic, mutagenic, teratogenic, atherogenic and oestrogenic effects in human and animals (Van Egmond, 1995). The illnesses caused by mycotoxins are called mycotoxicosis. The mycotoxins are not only hazardous to consumer health but also affect food quality resulting in huge economic losses for these countries (Moss, 1998).

The numbers of microorganisms on most dried fruits vary from a few hundreds per gram of fruits to thousands and they are mostly on the outer surfaces. Spores of bacteria and molds are likely to be the most numerous. When part of the fruit has supported growth and sporulation of mold before or after drying, mold spores may be present in large numbers. If drying trays are not clean and improperly loaded, a marked increase in the numbers of bacteria and fungi may take place during the drying process.

Spoilage of most dry fruits usually occurs during storage, handling and transport (Frazier and Westhoff, 1988).

Preservation of fruits by solar drying has been practiced for centuries. It is limited to climates with a hot sun and a dry atmosphere and to certain fruits, such as raisins, prunes, figs, apricots, pears and peaches. The fruits are spread out on trays and be turned during drying.

Although natural occurrence of mycotoxins and fungal contamination of many dried fruits were investigated in many parts of the world (Boyacioglu and Gonul, 1988; Steiner *et al*., 1988; Herry and Lemetayer, 1992; Zohri and Abdel-Gawad, 1993; Ozay *et al*., 1995; Elhalouat and Debevere, 1997; Elhalouat *et al*., 1998; Abdel-Sater and Saber, 1999 and Bayman, *et al.* 2002), none of these studies were reported in Yemen Republic. Therefore, this investigation was mainly planned to determine the mycoflora and mycotoxin contamination of some Yemeni dry fruits.

MATERIALS AND METHODS:

Collection of samples: Twenty dried samples (500 g each) of each of raisins, figs and dates fruits were collected from shops and markets of different sanitation levels at Sana'a, Taiz, Adan and Ibb governorates, Yemen Republic during the period from June 2002 to May 2003. Each sample was put in a sterile polyethylene bag, sealed and transferred to the laboratory. All samples were kept in a refrigerator (4ºC) till mycoflora and mycotoxin analysis.

Isolation of molds and yeasts: Fungi were isolated using the dilution plate method as described by Johnson and Curl (1972). Twenty five grams of each dried fruit sample was comminuted for 2 min in 250 ml of 0.12% sterile plain agar. Further dilutions were made and one ml of an appropriate final dilution was placed in each petridish. 1% glucose- and 40% sucrose-Czapek's agar media were used for isolation of glucophilic and xerophilic fungi, respectively. Chloramphenicol (20 µg/ml) and rose bengal (30 ppm) were used as bacteriostatic agents. Six plates were used for each sample (3 plates for each type of medium). The plates were incubated at 28ºC for 10 days. The developing fungi were counted and identified morphologically based on macro- and microscopic characteristics and by using the following references: Ainsworth & Bisby (1961), Booth (1971), Ellis (1971), Raper and Fennell (1977), Pitt (1979), Moubasher (1993), Samson *et al*. (1995) and Barnett & Hunter (1998)

Mycotoxins analysis: Twenty-five grams of each dried fruit sample was defatted by extraction with normal hexane for 10 h using Soxhlet-type extractor. The defatted residue was reextracted for another 10 h with chloroform. The chloroform extract was dried over anhydrous sodium sulphate, filtered and evaporated under vacuum to near dryness. The residue was diluted with chloroform to one ml.

Qualitative analysis of mycotoxins: The chloroform extracts were analyzed on precoated silica gel plate type 60F 254 (Merck) for the presence of different mycotoxins: Aflatoxins (B1, B2, G1 and G2), citrinin, sterigmatocystin, patulin, ochratoxin A, diacetoxyscirpenol, T-2 toxin and zearalenone toxin using thin layer chromatographic technique according to standard procedures (Scott *et al*. 1970, Thrane, 1986 and Van Egmond, 1995).

Quantitative determination of aflatoxins: The spots of aflatoxin B1 including the standards were removed from the plates, eluded with methanol and estimated spectrophoto-

metrically using Spectrophotometer (UNICAM Helios Gamma, Helios Delta) according to the methods described by Scott (1995).

Quantitative determination and confirmation of ochratoxin A: According to the method of Nesheim *et al.* (1976).

Bioassay of toxins: Brine shrimps (*Artemia salina* L.) were used for mycotoxins bioassay test according to the method described by Brown (1969) and Reiss (1993).

RESULTS AND DISCUSSION:

1-Glucophilic fungi (isolated on 1% glucose-Czapek's agar):

Data in Table (1) show that twenty one species and one variety belonging to 13 genera were collected from dried fruits of raisins (10 genera, 16 species +1 var.), figs (7 genera, 11 species) and dates (8 genera, 15 species + 1 var.) on 1% glucose-Czapek's agar at 28ºC. The gross total counts of glucophilic fungi in dried raisins, figs and dates were 2240, 640 and 1540 colonies per g dry weight in all samples tested respectively (Table 1). Most of the recovered fungi were isolated from dry fruits in many part of the world as reported by several investigators (Herry and Lemetayer, 1992; Zohri and Abdel-Gawad, 1993; Ozay *et al*., 1995; Elhalouat and Debevere, 1997; Abdel-Sater and Saber, 1999), but almost all of them were firstly isolated from dried raisins, figs and dates in Yemen.

***Aspergillus* was the most frequently isolated genus. It occurred in 70%, 30% and 75% of the samples comprising 63.4%, 59.4% and 71.4% of total fungi in dried raisins, figs and dates, respectively. Peter *et al.* (1990) studied the fungal contamination of fruits and vegetables and indicated that *Aspergillus* was isolated from 79.5% of the samples. In Egypt, Abdel-Sater and Saber (1999) found that *Aspergillus* was the predominant genus isolated from dried raisins and dates (90% and 100% of samples respectively), while Zohri and Abdel-Gawad (1993) found that *Penicillium* was the most predominant genus isolated from dried apricots, figs, prunes and raisins. Of the *Aspergillus*, 6 species and one variety were isolated of which *A. niger* and *A. flavus* were the most prevalent species followed by *A. fumigatus* and *A. ochraceus.* The remaining species were less frequently isolated and *A. versicolor* was only isolated from dried raisins. Most of the *Aspergillus* species recovered during the current investigation were isolated previously from different dried fruits in different parts of the world, but with variable frequencies and populations (Peter *et al.*, 1990; Zohri and Abdel-Gawad, 1993; Elhalouat and Debevere, 1997; Abdel-Sater and Saber, 1999).**

***Rhizopus stolonifer* was the second most common fungus isolated from dried fruits in Yemen. It occurred in 30%, 15% and 25% of the samples comprising 10.7%, 12.5% and 11.7% of total fungi in dried raisins, figs and dates, respectively (Table 1). It was not recorded by Abdel-Sater and Saber (1999) in their investigation and isolated in low frequency from two samples of dry raisins and one sample of each of dry figs and plums by Zohri and Abdel-Gawad (1993) in Egypt. Also, it was not isolated in Morocco by Elhalouat and Debevere (1997) from dried raisins and prunes.**

***Penicillium* was isolated from five samples of raisins (25%), four samples of dates (20%) and two samples of figs (10%). Similar finding was reported by Abdel-Sater and Saber (1999) in Egypt who found that *Penicillium* species isolated in low frequency from dry raisins (35%) and dates (30%). In contrast to our finding, Zohri and Abdel-Gawad (1993) found that *Penicillium* was the most prevalent genus and encountered from all samples of dry figs,**

prunes, apricots and raisins. It was represented by four species of which *P. chrysogenum* was the common species in the three dry fruits. This species was also the most common species recovered by Zohri and Abdel-Gawad (1993), Abdel-Sater and Saber (1999) in Egypt and by Elhalouat and Debevere (1997) in Morocco.

The remaining genera and species were isolated from one or two substrates with low frequency and total counts as represented in Table (1). Most of these fungi were isolated previously from various dry fruits, seeds and vegetables in many parts of the world as reported by several researchers (Herry and Lemetayer, 1992; Benkhemmar *et al*., 1993; Zohri and Abdel-Gawad 1993; Ozay *et al*., 1995; Elhalouat and Debevere 1997 and Abdel-Sater and Saber 1999).

Table (1): Average total counts (ATC, calculated per g dry weight of fruit) and number of cases of isolation (NCI) of various fungal genera and species isolated from dried fruits on 1% sucrose-Czapek's agar at 28ºC.

Genera & Species	Raisins		Figs		Dates	
	ATC	NCI	ATC	NCI	ATC	NCI
Alternaria alternate (Fries) Keissler	40	1	-	-	20	1
Aspergillus	1420	14	380	6	1100	15
A. flavus Link	420	8	120	2	180	4
A. flavus var. columnaris Raper & Fennell	20	1	-	-	20	1
A. fumigatus Fresenius	120	4	60	2	120	3
A. niger Van Tieghem	680	14	180	6	740	13
A. ochraceus Wilhelm	160	4	-	-	20	1
A. terreus Thom	-	-	20	1	20	1
A. versicolor (Vuill.) Tiraboschi	20	1	-	-	-	-
Cladosporium cladosporioides (Fres.) Mason & M.B. Ellis	60	3	20	1	-	-
Eurotium amstelodami Mangin	-	-	40	2	-	-
Fusarium oxysporum Schlecht	40	2	-	-	-	-
Gibberella fujikuroi (Sawada) Wollenw.	-	-	20	1	20	1
Mucor racemosus Fresenius	60	2	-	-	20	1
Nectria haematococca Barkeley & Brown	20	1	-	-	-	-
Nigrospora oryzae Hudson	40	1	-	-	20	1
Penicillium	260	5	80	2	160	4
P. chrysogenum Thom	220	4	60	2	120	2
P. citrinum Thom	20	1	-	-	-	-
P. funiculosum Thom	20	1	-	-	20	1
P. oxalicum Currie & Thom	-	-	20	1	20	1
Rhizopus stolonifer (Ehrenb.) Lind	240	6	80	3	180	5
Scopulariopsis brevicaulis (Sacc.) Bainier	60	2	-	-	20	1
Ulocladium atrum Preuss	-	-	20	1	-	-
Gross total counts	2240		640		1540	
Number of genera =13	10		7		8	
Number of species = 21+1 var.	16+1var.		11		15+1var.	

2-Xerophilic fungi (isolated on 40% sucrose-Czapek's agar):

Nine species belonging to seven genera were isolated from dried raisins, figs and dates on 40% sucrose-Czapek's agar at 28ºC, of which *Eurotium amstelodami*, *Zygosaccharomyces rouxii*, *Aspergillus niger* and *Penicillium chrysogenum* were the most prevalent fungi (Table 2). Elhalouat and Debevere (1997) isolated 7 spoilage molds and yeasts from dried raisins and prunes. They found that the predominant spoilage fungi were *Zygosaccharomyces rouxii*, *Eurotium amstelodami*, *Aspergillus niger*, *Penicillium chrysogenum* and *Fusarium* spp. In Egypt, Zohri and Abdel-Gawad (1993) found that *Euratium amstelodami*, *E. chevalieri*, *P. chrysogenum*, *A. niger*, *A. versicolor*, *A. wentii*

and *Cladosporium cladosporioides* were isolated in high frequency on 40% sucrose-Czapek's agar medium from dried apricots, prunes, figs and raisins.

***Eurotium amstelodami* was recovered from 60%, 30% and 20% of dried samples of raisins, dates and figs constituting 35%, 23% and 17% of total fungi, respectively. It was the most prevalent fungus isolated from dried raisins, while *A. niger* and *Z. rouxii* were more prevalent in dried figs and dates.**

***Zygosaccharomyces rouxii* is well known xerophilic fungus; emerging in 23%, 21% and 23% of dried samples of the three substrates in Yemen respectively. It was the most predominant fungus isolated from hydrated raisins and prunes in Morocco (Elhalouat and Debevere 1997), but it was not reported in Egypt from dried raisins, figs, dates, apricots and plums (Zohri and Abdel-Gawad, 1993, Abdel-Sater and Saber, 1999).**

***Aspergillus niger* was the most predominant fungus isolated from dried dates on 40% sucrose-Czapek's agar medium. It was recovered from 45%, 40% and 30% of dried samples of dates, raisins and figs respectively. *P. chrysogenum* was also isolated in high frequency from raisin samples (40%) and in low frequency from date samples (10%). Abdel-Hafez *et al.* (1990) rated most of these fungi as osmotolerant, but with variable degrees (osmotic potential ranging from -0.32 to -4.64 MPa on sucrose-Czapek's medium). The remaining genera and species were isolated from one or two substrates with rare frequency of occurrence as shown in Table (2).**

Table (2): Average total counts (ATC, calculated per g dry weight of fruits) and number of cases of isolations (NCI) of various fungal genera and species isolated from dried fruits on 40% sucrose-Czapek's agar at 28ºC.

Genera & Species	Raisins		Figs		Dates	
	ATC	NCI	ATC	NCI	ATC	NCI
Aspergillus	140	8	110	6	220	9
A. flavus Link	20	1	20	1	40	2
A. fumigatus Fresenius	20	1	10	1	20	2
A. niger Van Tieghem	100	8	80	6	160	9
Cldosporium cladosporioides (Fresen.) Mason & M.B. Ellis	80	4	-	-	20	1
Eurotium amstelodami Mangin	340	12	60	4	120	6
Humicola minima Fassatiova	-	-	-	-	20	1
Penicillium chrysogenum Thom	160	8	100	5	40	2
Rhizopus stolonifer (Ehrenb.) Lind	20	1	-	-	-	-
Zygosaccharomyces rouxii (Boutroux) Yarrow	220	10	80	6	110	6
Total counts	960		350		530	
Number of genera=7	6		4		6	
Number of species=9	8		6		8	

3-Mycotoxin contamination of dried fruits:

Thin layer chromatographic analysis of 60 samples tested of dried raisins, figs and dates (20 each), showed that six samples of dried raisins and two samples of each of dried figs and dates were naturally contaminated with aflatoxin B1. The concentrations of aflatoxin were ranged between 130-350 µg/kg of raisins, 120-250 µg/kg of figs and 110-180 µg/kg of dates. In France, Herry and Lemetayer (1992) reported that aflatoxin B1 was detected in dried raisins fruit collected from shops and supermarkets during 1989-1990. Ozay *et al.* (1995) recorded the presence of aflatoxin in dried figs and raisins in Turkey. Akerstrand and Moller (1989) reported that out of 103 samples of fig collected in Sweden, 53 samples

were contaminated with aflatoxin and the concentration of aflatoxin ranged from 5-203 µg/kg. In Egypt, Abdel-Sater & Saber (1999) surveyed the presence of aflatoxin in 60 samples of dried raisins, dates and figs. They found that aflatoxin B1 was detected in one sample of raisin (550 µg/kg), 2 samples of dates (300-390 µg/kg) and 5 samples of figs (600-780 µg/kg). In the other hand, Stoloff, (1976) recorded no detectable aflatoxin in 108 samples of Raisins and 62 samples of dates in USA, while only 3-6% of tested figs examined (165 samples) contained aflatoxin in the range of 2-29 µg/kg.

The results of thin layer chromatographic analysis of chloroform extracts of 60 samples of dried fruits revealed that two samples of dried figs were naturally contaminated with ochratoxin A at levels between 70 to 160 µg/kg of fig. Similar to our findings, Ozay *et al.*(1995) recorded the presence of ochratoxin A in dried figs in Turkey. In USA, Bayman, *et al.* (2002) examined 50,000 figs for fungal infections and measured ochratoxin content in figs with visible fungal colonies. Pooled figs infected with *Aspergillus alliaceus* contained ochratoxin A, figs infected with the *A. ochraceus* group had little or none, and figs infected with *Penicillium* had none. In Egypt, Zohri and Abdel-Gawad (1993) reported that all the samples tested of dried fruits were contaminated by ochratoxin A and the concentrations ranged between 50-110 µg/kg of apricots, 60-120 µg/kg of figs and 210-280 µg/kg of prunes. They also found that all samples of raisins were naturally free from mycotoxins. Abdel-Sater and Saber (1999) recorded a detectable amount of ochratoxin A in two samples of dates in Assiut, Egypt (360-450 µg/kg). Ochratoxin A is a secondary metabolite produced by several species of *Aspergillus* and *Penicillium* that has been found in a wide variety of cereal grains, coffee beans, cocoa, beer, red wine and recently found in raisins produced in several countries (Trucksess *et al*., 1999). It also has been identified in tissues and blood of animals fed contaminated feed and in human blood in Balkans, Scandinavia, Germany, France and Canada. It is nephrotoxic to all animal species studied so far, teratogenic, immunotoxic, genotoxic, mutagenic and carcinogenic which lead to life-threatening pathologies (Creppy, 1999).

Results of the brine shrimp bioassay revealed that nearly 25% of all samples were toxic to brine shrimp *Artemia salina* larvae (Table 3). Similar observation was reported by Abdel-Sater and Saber (1999) in Egypt who found that 30% of dried raisins, dates and figs samples were proved to be toxic to the test organism *Artemia salina*. High ratio of toxicity using brine shrimp bioassay test may be due to that some fungi elaborate naturally occurring fatty acids that are toxic to brine shrimp. Thus toxicity towards brine shrimp should be confirmed with at least additional test organism like chicken embryos (Curtis *et al*. 1974).

CONCLUSION:

The present work indicated that dried fruits examined were contaminated with several glucophilic and xerophilic fungi especially members of *Aspergillus, Penicillium, Eurotium, Zygosaccharomyces* and *Rhizopus*. Many of these fungi are capable of producing mycotoxins such as aflatoxin B1 and ochratoxin A. These findings indicate that there may be a risk of human exposure to mycotoxins through the consumption of dry fruits or juices and jams manufactured with dried fruits. So, strict hygiene microbiological measured must be applied during different steps during harvest, handling, transport, storage and drying to avoid contamination of dried fruits by mycoflora and mycotoxins which are harmful to human health.

Table (3) Number of dried fruits samples contaminated with mycotoxins out of 20 and the toxicity of their extracts against *Artemia salina* larvae.

Samples	Number of contaminated samples	Mycotoxins detected by TLC analysis	Concentration (µg/kg)	Bioassay toxicity test (*A. salina*)
Dried raisins	6	Aflatoxin B1	130-350	8
Dried figs	2 2	Ochratoxin A Aflatoxin B1	70-160 120-250	4
Dried dates	2	Aflatoxin B1	110-180	3

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Ass. Univ. Bull. Environ. Res. Vol. 7 No. 2, October 2004

الفلورا الفطرية والسموم الفطرية الملوثة لبعض الثمار الجافة فى اليمن

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استهدف البحث التعرف على الفلورا الفطرية المصاحبة لعدد ٦٠ عينة من الثمار المجففة فى اليمن، وهى الزبيب، التين والبلح، والتى تم جمعها من أسواق مختلفة بمحافظات صنعاء، تعز، عدن وأب. وقد تم عزل وتعريف ٢٣ نوعا فطريا وصنف واحد تنتمي إلى ١٥ جنساً من العينات المختبرة على نوعين من الأوساط الغذائية. وكانت أكثر الأنواع الفطرية شيوعاً وتعداداً على وسط الغذائى ١% جليكوز شابكس–أجار والتحضين عند ٢٨ ° م هى أسبرجلس أنواع نيجر، فلافس، فيوميجاتس، أوكروشيس، بنيسيليوم كريزوجينيم وريزوبس ستولنيفير. أما على الوسط الغذائى ٤٠% سكروز شابكس–أجار فكانت أكثرها شيوعاً هى أيروتيم أمستيلودامى، وزيجوساكروميسس روكسى، أسبرجلس نيجر وبنيسيليوم كريزوجنيم.

كما تناول البحث تحليل مستخلصات عينات الثمار المجففة (٦٠ عينة) باستخدام T.L.C. للكشف عن تلوثها بالسموم الفطرية وقد وجد أن مستخلصات ٦ عينات من الزبيب المجفف و مستخلص عينتان من كل من البلح والتين ملوثة طبيعياً بسموم الأفلاتوكسينات من النوع B1 وبتراكيز مختلفة تتراوح ما بين ١٣٠–٣٥٠ ميكروجرام لكل كيلوجرام من الزبيب ، ١٢٠–٢٥٠ ميكروجرام لكل كيلوجرام من التين وتركيز ١١٠–١٨٠ ميكروجرام لكل كيلوجرام من البلح المجفف. كما تم التعرف على وجود سم الأوكراتوكسين فى ٤ عينات من التين المجفف بتركيز يتراوح ما بين ٧٠–١٦٠ ميكروجرام لكل كيلوجرام من التين. ولم يتم إثبات وجود السموم الفطرية الأخرى فى العينات المختبرة تحت الدراسة.

كما تم تحليل تواجد السموم الفطرية فى عينات الثمار المجففة بطريقة التحليل الحيوية البيولوجية باستخدام يرقات كائن بحرى *Artemia salina* وثبت أن المستخلص الكلورفورمى لـ ١٤ عينة من الثمار المجففة سامة ليرقات الكائن البحرى بدرجات متفاوتة.