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Fungal Contamination and Aflatoxins Content of Dry Raisins Fruits in Sana'a City, Republic of Yemen

تلوث الزبيب المجفف بالفطريات وسموم الأفلاتوكسين فى مدينة صنعاء، الجمهورية اليمنية

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ABSTRACT: This study was designed to study the mycoflora and aflatoxin content of dry raisins in Yemen Republic. Thirty six raisin samples collected from different shops and markets in Sana'a city were analyzed mycologically for the presence of fungi. A total of forty eight species belonging to 20 genera were recovered from the analyzed raisins samples on three cultural media. *Aspergillus* was the most dominant genera on the three type of media, of which *A. niger* was the most common species. *A. flavus* was isolated in moderate, low and rare frequency on 1% and 20% sucrose Czapek's and Sabouraud dextrose agar media. *Penicillium* was isolated in moderate frequency on 1 and 20% sucrose Czapek's agar media, but in low frequency on Sabouraud dextrose agar medium. The raisin samples were analyzed for the presence of total aflatoxin using ELISA technique. The results revealed that 3 out of 7 samples of raisins analyzed were contaminated with total aflatoxin at levels ranged from 2678.66 to 11556.88 ppt (ng Kg^{-1}).

Keywords: *dry raisin, fungi, Aspergillus flavus, aflatoxins, Yemen.*

المستخلص: تم فى هذه الدراسة تشخيص المحتوى الفطرى وكمية سموم الأفلاتوكسين الكلي للزبيب المجفف فى الجمهورية اليمنية، حيث تم جمع ستة وثلاثون عينة زبيب مجفف من مختلف المحلات والأسواق فى مدينة صنعاء من مختلف الأنواع والمصادر، وتم تحليلها لعزل الفطريات المتواجده عليها بإستخدام ثلاثة أوساط غذائية. تم عزل ثمانية وأربعون نوعاً فطرياً تنتمى الى 20 جنس من الزبيب المجفف فى الأوساط الغذائية الثلاثة، وكان جنس الأسبرجلس أكثر الأجناس شيوعاً بينما كان النوع أسبرجلس نيجر أكثر الأنواع عزلاً من هذا الجنس. تم عزل فطر أسبرجلس فلافس بمعدل متوسط فى وسط 1% سكروز شبك أجار وبمعدل منخفض فى وسط 20% سكروز شبك أجار بينما عزل بشكل نادر فى الوسط الغذائى سابورود دكستروز أجار. أما بالنسبة لفطر البنسليوم فقد عزل بمعدل متوسط فى أوساط 1% و 20% سكروز شبك أجار وعزل بمعدل منخفض من وسط سابورود دكستروز أجار. تم تحليل عينات الزبيب المجففة لتواجد سموم الأفلاتوكسين بواسطة تحديد الكم الكلى للأفلاتوكسين بإستخدام طريقة الأليزا والتى تم بواسطتها التأكد من تلوث 3 عينات من أصل 7 عينات زبيب مجفف بسموم الأفلاتوكسين بمعدل يتراوح بين 2678.66- 11556.88 جزء من التريليون (نانوجرام لكل كيلوجرام) من الزبيب المجفف.

كلمات مدخلية: *الزبيب المجفف، الفطريات، Aspergillus flavus، سموم الأفلاتوكسين، اليمن.*

INTRODUCTION

In most developing countries, agriculture is the backbone of the economy and export crops are greatly depended upon for 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). Foodstuffs for human consumption are spoiled not only because of degradation by microorganisms (aerobic decomposition and anaerobic putrefaction) but also through contamination with toxin-producing bacteria and fungi. Some fungi produce mycotoxins, of which aflatoxins have become the most widely known (Schlegel, 1996). In the range of low and intermediate water activity of food (a_w), yeasts and moulds are the common spoilage flora. Immediately after harvest, the dominant species are field fungi like yeasts, *Fusarium* and *Penicillium spp.* During storage, the field fungi are gradually substituted by the storage fungi like *Eurotium* (*Aspergillus glaucus* group), *A. niger* and *Penicillium spp.* (Wallace, *et al.* 1976).

Storage conditions are one of the factors that determine the type and quantity of fungal contamination of fruits (Lillehoj, *et al.* 1980; Hill and Lacey, 1983). The general sun-drying process that is used, i.e., picking partially dried fruits from the ground and exposing them to direct sunlight until dehydration is completed, dramatically increase the potential for "*Aspergillus*" fungal contamination, resulting in greater changes of aflatoxin development (Tosun and Delen, 1998).

Mycotoxins are secondary metabolites of fungi that are toxic to higher animals. They are typically low molecular weight compounds with diverse structures (Shibamoto, 1998). They contaminate many food products, especially those undergoing long-term storage such as peanuts, grains, and cereals. Moulds of the genera *Aspergillus*, *Penicillium* and *Fusarium* produce 80% of the total toxic substances (Hesseltine, 1974). Aflatoxins are produced by certain strains of *A. flavus*, *A. parasiticus* and *A. nomius*, which is phenotypically similar to *A. flavus* but with a distinctive bullet shaped sclerotia (Fente, *et al.* 2001). The same authors reported that other species could produce aflatoxins in minute quantities include *A. peudotamarii*, *A. bonbysis* and *A. ochraceoroseus*.

The aim of the present study was to isolate and identify spoilage fungi contaminating dried raisin fruit and to determine the total aflatoxins contents of dry raisins fruits in Sana'a city, Yemen.

MATERIALS AND METHODS

Collection of Samples

Thirty six samples (250 g each) of raisins were collected from different markets and shops at Sana'a city, Republic of Yemen, during 2005. Kind, number, and source of production of the collected samples are shown in Table (1). Each sample was placed in a sterile polyethylene bag, sealed, put in another bag, and transferred to the laboratory for mycoflora and mycotoxin analysis.

Table 1. List of dried raisin samples analyzed throughout this study.

Kind of raisins	Source of raisins	No. of samples tested
White raisin	Yemen	23
	China	4
	Iran	1
	Syria	1
Black raisin	Yemen	7
Total		36

Isolation and Identification of Fungi

Fungi were isolated using the dilution plate method as described by Johnson and Curel (1972). Twenty-five g of each sample were suspended into 250 ml of sterile physiological solutions (0.85% NaCl) in a sterile 500 ml conical flask. The flasks were shaken, using a mechanical shaker for 20-30 minutes. Dilutions from 10^{-1} to 10^{-3} were made under aseptic conditions. One ml of appropriate dilution was transferred into Petri dish. For each sample, nine Petri dishes were used (three for every medium). Twenty ml of melted agar media were added after being cooled to 45-50°C. The Petri dishes were incubated for 7 days at 28°C.

Isolation Media

The culture media used for isolation of fungi were 1% sucrose Czapek's agar medium, 20% sucrose Czapek's agar medium and Sabouraud dextrose agar medium. The pH of all media was adjusted to 5.5. These media were sterilized by autoclaving at 121°C and 1.5 bar for 30 minutes. Chloramphenicol (500 mg l^{-1}) was added to the

medium after sterilizing as bacteriostatic agent.

The developing fungal colonies were counted and identified up to genus and species level based on macroscopic and microscopic characteristics. The identification of fungal genera and species was made with the help of the following references: Booth (1971) for the genus *Fusarium*, Ellis (1971) for dematiaceous hyphomycetes, Raper and Fennell (1977) for the genus *Aspergillus*, Pitt (1979) for the genus *Penicillium* and its teleomorphic states *Eupenicillium*, and *Talaromyces*, Moubasher (1993) for fungi in general, and Samson, *et al.* (1995) for fungi in general.

Potato dextrose agar medium was used for purification of fungi. The purified fungi were transferred to slants of the same medium for the good sporulation, and kept in refrigerator at 4°C.

Determination of Total Aflatoxins in Dried Raisin Samples

Seven raisin samples were analyzed to determine their content of total aflatoxins by enzyme-linked immunosorbent assay (ELISA) technique using R-biopharm-Germany kits (RIDASCREEN FAST), manufactured by R-Biopharm Company, Germany (Sinha, *et* al. 1993). The samples were analyzed in Laboratory of Molecular Neurology and Functional Neuroproteomics, Swiss Federal Institute of Technology Lausanne (EPFL), Switzerland.

RESULTS

Fungi isolated from raisins on 1% sucrose Czapek's agar

Thirty-six species belonging to 18 genera were isolated from raisin samples on 1% sucrose Czapek's agar medium. The total fungal count was 14560 per g in all samples (Table 2). *Aspergillus* was the most frequent genus, occurred in 88.88% of samples comprising 73.54% of total count of fungi. *A. niger* was present in high frequency representing 70.31% of total count of fungi. *A. flavus* occurred in moderate frequency in 33.33% of samples. *A. terreus* was present in low frequency. *A. fumigatus, A. parasiticus* and *A. wentii* were isolated in rare frequency. *Penicillium* was recovered in moderate frequency representing 13.32% of total count of fungi in all samples. *Cladosporium, Curvularia lunata, Fusarium, Phoma and, Rhizopus,* were isolated in low frequency comprising 7.43, 0.77, 0.25, 0.45 and 0.64%, respectively of total count in all samples. *Drechslera poae, Emericella nidulans, Glicoladium virido, Myrothecium roridum, Scopulariopsis brevicaulis, Setosphaeraia rostrata,* sterile mycelium, *Taeniolla exillis, Tetracoccosporium paxinum, Ulocladium* and yeasts were isolated in rare frequency.

Fungi isolated from raisins on 20% sucrose Czapek's agar

Twenty-four species belonging to 10 genera were isolated from raisin samples on 20% sucrose Czapek's agar medium. The total fungal count was 16910 per g in all samples (Table 2). *Aspergillus* was the most common genus, occurred in 91.66% of samples comprising 74.21% of total count of fungi. *A. niger* was the most common species isolated in high frequency represent 74.21% of total count, whereas, *A. flavus* was isolated in low frequency. *Aspergillus terreus, A. wentii,* and *A. versicolor* were recovered in rare frequency.

Penicillium and *Rhizopus stolonifer* were isolated in low and moderate frequency, respectively, comprising 9.22 and 0.47% of total count of all samples. *Alternaria, Cladosporium, Cochliobolus sativus, Curvularia lunata, Fusarium oxysporum, Phoma sp. Rhizopus stolonifer,* Sterile mycelium, *Ulocladium chartarum,* and yeasts were isolated in rare frequency.

Fungi isolated from raisins on Sabouraud dextrose agar

Twenty species belonging to 8 genera were isolated from raisin samples on Sabouraud dextrose agar medium. The total count of fungi was 14860 per g in all samples (Table 2). *Aspergillus* was the most common genus, occurred in 80.55% of samples comprising 68.23% of total count of fungi. *A. niger* was the most common species representing 64.26% of total count, whereas *A. flavus* were occurred in low frequency. *A. fumigatus, A. terreus, A. ustus*, and *A. tamarii* were recovered in rare frequency. *Rhizopus stolonifer* was isolated in moderate frequency comprising 3.09% of total count. *Penicillium* and *Phoma sp.* were isolated in low frequency comprising 8.54 and 2.82, respectively of total count. *Alternaria alternate, Cladosporium, Humicola insolens,* Sterile mycelium, and yeasts were occurred in rare frequency.

Table 2. Total counts (TC calculated X10 g^{-1} of raisin sample), number of cases of isolation (NCI) and occurrence remarks (OR) of fungal genera and species recovered from 100 dried fruit samples on 1 and 20% sucrose Czapek's and Sabouraud dextrose agar medium at 28°C.

Fungal genera and species	1% sucrose Czapek's			20% sucrose Czapek's			Sabouraud dextrose		
	TC	NCI	OR	TC	NCI	OR	TC	NCI	OR
Alternaria	2	2	R	12	3	R	1	1	R
A. alternata	2	2	R	2	2	R	1	1	R
A. macrospora	-	-	-	10	1	R	-	-	-
Aspergillus	1137	32	H	1265	33	H	1014	29	H
A. flavus	13	12	M	6	5	L	23	3	R
A. fumigatus	20	1	R	-	-	-	10	1	R
A. niger	1087	31	H	1256	30	H	955	28	H
A. parasiticus	1	1	R	-	-	-	-	-	-
A. tamarii	-	-	-	-	-	-	3	2	R
A. terreus	4	4	L	1	1	R	12	3	R
A. ustus	-	-	-	-	-	-	1	1	R
A. wentii	2	1	R	1	1	R	-	-	-
Aspergillus sp.	10	1	R	1	1	R	10	1	R
Cladosporium	115	5	L	31	2	R	171	3	R
C. cladosporiodes	3	2	R	1	1	R	1	1	R
C. herbarum	2	2	R	-	-	-	-	-	-
C. oxysporum	110	1	R	-	-	-	90	2	R
C. sphaerospermum	-	-	-	30	1	R	80	2	R
Cochliobolus	2	2	R	1	1	R	-	-	-
C. sativus	1	1	R	1	1	R	-	-	-
C. spicifer	1	1	R	-	-	-	-	-	-
Curvularia	12	6	L	7	3	R	-	-	-
C. lunata	12	6	L	7	3	R	-	-	-
Drechslera poae	1	1	R	-	-	-	-	-	-
Emericella nidulans	1	1	R	-	-	-	-	-	-
Fusarium	4	4	L	2	2	R	-	-	-
F. oxysporum	2	2	R	2	2	R	-	-	-
Fusarium sp	2	2	R	-	-	-	-	-	-
Gliocladium virido	1	1	R	-	-	-	-	-	-
Humicola insolens	-	-	-	-	-	-	1	1	R
Myrothecium roridum	1	1	R	-	-	-	-	-	-
Penicillium	205	14	M	156	10	M	147	7	L
P. aurantiogriseum	-	-	-	-	-	-	1	1	R
P. camemberti	1	1	R	-	-	-	-	-	-
P. chrysogenum	-	-	-	2	1	R	-	-	-
P. citrinum	4	1	R	5	1	R	-	-	-
P. corylophilum	-	-	-	3	2	R	1	1	R
P. cyaneum	-	-	-	-	-	-	1	1	R
P. glabrum	-	-	-	1	1	R	21	2	R
P. griseofulvum	-	-	-	2	1	R	1	1	R
P. italicum	-	-	-	93	3	R	51	2	R
P. patulum	-	-	-	1	1	R	-	-	-
P. urticae	-	-	-	1	1	R	-	-	-
P. variabile	-	-	-	45	4	L	51	3	R
P. vinaceum	-	-	-	1	1	R	-	-	-

Table 2. Cont.

Fungal genera and species	1% sucrose Czapek's			20% sucrose Czapek's			Sabouraud dextrose		
	TC	NCI	OR	TC	NCI	OR	TC	NCI	OR
Penicillium sp.	16	5	L	2	2	R	20	1	R
Phoma	7	6	L	73	5	L	42	4	L
P. glomerata	1	1	R	-	-	-	-	-	-
Phoma sp.	6	6	L	73	5	L	42	4	L
Rhizopus	10	7	L	8	7	L	46	10	M
R. stolonifer	9	6	L	8	7	L	46	10	M
Rhizopus sp.	1	1	R	-	-	-	-	-	-
Scopulariopsis brevicaulis	1	1	R	-	-	-	-	-	-
Setosphaeria rostrata	1	1	R	-	-	-	-	-	-
Stemphylium variabile	-	-	-	-	-	-	1	1	R
Sterile mycellium	31	3	R	141	3	R	70	2	R
Taeniolla exillis	1	1	R	-	-	-	-	-	-
Tetracoccosporium paxinum	1	1	R	-	-	-	-	-	-
Ulocladium	4	3	R	2	2	R	-	-	-
U. alternariae	1	1	R	-	-	-	-	-	-
U. atrum	1	1	R	-	-	-	-	-	-
U. botrytis	2	1	R	-	-	-	-	-	-
U. chartarum	-	-	-	2	2	R	-	-	-
Yeast	6	3	R	4	2	R	13	2	R
Total Count	**1456**	-	-	**1691**	-	-	**1486**	-	-
Number of Genera	**18**	-	-	**10**	-	-	**8**	-	-
Number of Species	**36**	-	-	**24**	-	-	**20**	-	-

H= High occurrence; from 15- 30 samples; M= moderate occurrence; from 8-14 samples;
L= low occurrence; from 4-7 samples; R= rare occurrence; from 1-3 sample

Aflatoxin contamination of raisin samples

Analysis of raisin samples for the natural occurrence of total aflatoxin using ELISA technique shows that 3 out of 7 samples of raisins were contaminated with total aflatoxin at levels 2678.66-11556.88 ppt (ng Kg^{-1}) (Table 3).

Table 3. Total aflatoxin content of dried raisin samples.

Sample no.	Sample source	Total Aflatoxins (ppt)
1	Yemen	0
2	China	2678.66
3	Yemen	0
4	Yemen	4222.92
5	Yemen	0
6	Yemen	11556.88
7	Yemen	0

DISCUSSION

Results presented in Table (2) showed that *Aspergillus* was the most predominant genus isolated from raisins on 1% and 20% sucrose Czapek's and sabouraud dextrose agar media. It represents 73.54, 74.21 and 68.23% of total count of fungi. Abdel-Sater and Saber (1999) isolated 8 genera, 15 species and 1 variety from raisins samples. They found that *Aspergillus* and *Eurotium* were the predominant genera, whereas *Penicillium* was isolated in moderate frequency. Abrunhosa, *et al.* (2001) isolated eight genera of fungi from grapes samples harvested from two regions in Portugal. *Penicillium, Cladosporium,* and *Botrytis* prevailed in the Douro region whereas *Cladosporium* and *Botrytis* were dominant in the Vinhos Verdes region. Abarca, *et al.* (2003) found that fungal contamination was detected in 49 of 50 samples of dried vine fruits. They also found

that *A. niger* var *niger* was isolated from 98% of samples, and *A. carbonarius* was found in 58% of the samples. Magnoli, *et al.* (2003) studied the mycoflora of grapes. They indicated the presence of seven genera of filamentous fungi. *Alternaria spp.* were the most frequent mould occurred in 80% of investigated samples followed by *Aspergillus spp.* Magnoli, *et al.* (2004) surveyed the mycoflora of dried vine fruits and identified nine genera of filamentous fungi. Among them *Aspergillus spp.* were the most frequent mould in these samples. Serra, *et al.* (2005) found that the most frequently detected genera found in grapes were *Cladosporium*, *Penicillium*, *Botrytis* and *Aspergillus*. In addition, they detected that the mycoflora of grapes is composed of common field fungi, such as *Alternaria*, *Cladosporium* and *Aureobasidium*, as well by pathogenic agents of grapes such as *Aspergillus*, *Botrytis*, *Penicillium* and *Rhizopus*.

Lozada (1995) reported that fruits contamination by different moulds occurs during preharvesting, harvesting and grape processing. During these periods, temperature and humidity are important factors in mycelial growth and conidia germination. The composition of the fruit influences the likely type of spoilage. Because most fruits are somewhat acid, dry at the surface, and deficient in B vitamins, moulds are the most common causes of spoilage. The composition, too, must determine the particular kinds of moulds most likely to grow; thus, some kinds of fruits support a large variety of spoilage organisms and other kinds comparatively few (Frazier and Westhoff, 2000). Wareing, *et al.* (2001) reported that mould growth is heavy when drying times are extended during the rainy season. This may be as a result of increased relative humidity during the rainy season, or that products take longer to dry if rewetted. Bennett and Klick (2003) reported that filamentous fungi can cause spoilage of grapes and/or contaminated them with toxic secondary metabolites named mycotoxins.

The results in Table (3) show that 3 out of 7 raisin samples were contaminated with total aflatoxins at levels 2678.66-11556.88 ppt (ng kg^{-1}). Abdel-Sater and Saber (1999) analyzed dried raisin samples for the presence of aflatoxins by chromatographic analysis. Aflatoxin B_1 was detected in one sample out of 20 tested at levels of 550 $\mu g\ kg^{-1}$. Youssef, *et al.* (2000) examined 100 dried-raisin samples for mycotoxin contamination. Aflatoxin B_1 was detected in two of these samples at concentrations of 220 and 300$\mu g\ kg^{-1}$. Zohri and Abdel-Gawad (1993) analyzed samples of raisins for the natural occurrence of aflatoxins. They found that all raisins samples were free of mycotoxins.

The highest concentrations of aflatoxins are produced as a result of post-harvest spoilage of commodities stored under warm moist conditions. Significant concentrations may also be produced in the field before harvest. This arises from endophytic association between these moulds and plants, such as maize and groundnut (Hill, *et al.* 1985). The toxigenic strains of *Aspergillus flavus* are distributed worldwide in soil and air and have been reported to contaminate a variety of foods and feeds (Bilgrami, 1984; Mahmoud, 1993). Firm and ripe fruits show little contamination when they are dried immediately. Sales, *et al.* (2005) found that the presence of *A. flavus* on food-contact surfaces and in the air surrounding the production area for dried Cavendish bananas is indicative of high probability for Philippine dried Cavendish banana chips to be contaminated with aflatoxigenic fungi and aflatoxins.

The Yemeni limit standard for total aflatoxin is 20 $\mu g\ g^{-1}$ for various foodstuffs (Yemeni Standard Limits, 2001). World Health Organization (WHO) standards for aflatoxin B_1 in various foodstuffs is 5 ng $g^{-1}$and the total aflatoxin level can not exceed 10 ng g^{-1}. Germany, Switzerland, USA, and Hungary limit 4, 5, 20 and 5 ng g^{-1} for various foodstuffs, respectively (Papp, *et al.* 2002). Current legislation limits 4$\mu g\ kg^{-1}$ for total aflatoxins in dried fruits for direct human consumption and 10$\mu g\ kg^{-1}$ to be subjected to sorting or other physical treatment before consumption or use as an ingredient in foodstuff (Commission Regulation, 2003).

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