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Mycoflora and Mycotoxigenic Moulds of Pistachio Nuts for Human Consumption in the Sultanate of Oman

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الفلورا الفطرية والفطريات المفرزة للسموم في جوزة الفستق المعد للاستهلاك في سلطنة عمان

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خلاصة : أجري هذا البحث لدراسة الفلورا الفطرية المتواجدة في جوزة الفستق المعد للاستهلاك . وقد تم الكشف على ٧٥ علبة مختلفة من الفستق تسوقها شركات مختلفة في سلطنة عمان ولقد تم عزل عدد من الفطريات بنسب التلوث التالية :
Aspergillus niger 14.8%, Penicillium spp. 13.6%, A.flavus 9.7%, A. nidulans 1.6 %, and < 1% for Cladosporium cladosporoides, Alternaria alternata, Aspergillus ochraceus, and Ulocladium consortiale.
اثبتت نتائج هذا البحث ان هنالك اختلافات كبيرة في جودة الأنواع المختلفة التي تسوقها الشركات . كما وجد أفلاتوكسن ب المسبب للسرطان في أحد الأنواع (من بين خمسة عشر نوعا تمت دراستها) بمقدار ٢ ميكروجرام / كيلوجرام .

ABSTRACT: Mycoflora of 75 cans of different batches and brands of pistachio nuts purchased in the Sultanate of Oman was studied. A number of fungi were isolated, with percentages of contaminated nuts [average between brands] as follows : *Aspergillus niger* 14.8%, *Penicillium* spp. 13.6%, *A. flavus* 9.7%, *A. nidulans* 1.6% and <1% for *Cladosporium cladosporoides, Alternaria alternata, A. ochraceus* and *Ulocladium consortiale*. Significant differences were found among the batches and brands contaminated by *A. flavus*. Aflatoxin B_1 was found only in one sample out of 15 assayed at a level of 2 ug/kg.

The endocarp (the shells) of pistachio fruits (*Pistacia vera* L.) naturally splits at maturity along its suture, a highly desirable trait as the nuts are usually marketed in the shell to be opened by hand. Contamination of pistachio nuts by toxigenic fungi is most likely to occur in the field as well as during poor storage and processing conditions. Herperkan *et al.* (1994) reported that mould count of pistachio nuts surveryed in Turkey was in the range of 10^3 -10^4 cfu g^{-1} and 10^5 - 10^6 cfu g^{-1} for harvest and storage samples respectively. Doster and Michailides (1994) reported a total of 14 *Aspergillus* species isolated from pistachio nut from 11 commercial orchards in California, USA. Schatzki and Pan (1996) measured the distribution of aflatoxins concentration in 19 processed streams of pistachio nuts in USA and concluded that all aflatoxins found arise in the orchard. It appears that the source of contamination of pistachio nut in the orchards is due to the contamination and sporulation of fungi in pistachio litter (Doster and Michailides, 1994).

Freshly harvested nuts are usually dehulled manually or mechanically. Most countries dry the nuts while they are in the hull, store them for sometime, and later soak them in water to ease the removal of the hulls (Woodroof, 1979). Depending upon the environmental conditions of nut processing contamination may occur. The probability of the contamination of pistachio nuts during storage and distribution is higher than other nuts that do not split open at maturity (Herperkan *et al.*, 1994). Mycotoxins are a group of highly poisonous metabolites produced by different fungi growing on crops or their products. They were isolated from different sources in different parts of the world including food and feed (Sanchis *et al.*, 1986), wheat (Abramson *et al.*, 1990), nuts and oilseeds (Pitt *et al.*, 1993) and groundnut (Awuah and Kpodo, 1996). In the Arabian countries mycotoxins were studied in a number of crops including rice (Abdel-Hafez *et al.*, 1987); hazelnut and walnut (Abdel-Hafez and Saber, 1993), dried fruits (Zohri and Abdel-Gawad, 1993). And tobacco (El-Maghraby and Abdel-Sater, 1993). Aflatoxins which are potentially carcinogenic metabolites of *A. flavus* and *A. parasiticus* have been isolated from a number of sources in the Arabian countries that include groundnuts (Hag Elamin *et al.*, 1988), oilseeds (Elshafie *et al.*, 1991); rotted lemon (Mahmoud and Omar, 1994), onion (Zohri and Abdel-Gawad, 1993), and human and camel milk (Saad *et al.*, 1989, 1995).

Little information has been published on mycotoxins and mycoflora of pistachio nuts (Mojtahedi *et al.*, 1979; Bilgrami, 1994; Doster and Michailides, 1994; Herperkan *et al.*, 1994; Schatzki and Pan,1996). Most of the studies dealt with pistachio nuts in conjunction with other kinds of nuts (Abdel-Gawad and Zohri, 1993; Jimenez *et al.*, 1991; Steiner *et al*, 1992; Tabata *et al.*, 1993).

In the Sultanate of Oman pistachio nuts are a popular snack, eaten salted and roasted and are traditionally added to Omani halwa (sweet) which is consumed daily with coffee by most families. Shelled and split-shells of pistachio nuts have a good market and they are sold unpacked or in cans under various brand names. The mycoflora and mycotoxins of pistachio nut have not previously been investigated in the Sultanate of Oman. This work investigates the frequency of the mycotoxigenic fungi of samples from different brands and batches of pistachio nuts purchased in retail markets in Muscat area, Sultanate of Oman, that were ready for consumption. Little emphasis has been put on the distribution of the various mycotoxins, instead, only aflatoxin has been investigated. It is believed that it is a more rational approach to identify the mycoflora and mycotoxigenic species of a sample rather than the determination of the hundreds mycotoxins by methods that differ greatly, and others that still need to be developed (Chang-Yen and Bidasee, 1992).

Materials and Methods

SAMPLE COLLECTION: In the present study five known commercial brands of pistachio nuts available in the retail markets that were ready for human consumption were investigated. Four were vacuum packed while the fifth was unpacked, cheaper and sold by weight. Three batches of each brand having different production and expiry dates were purchased. Each batch consisted of five cans of nuts each containing 140 g of nuts The nuts were unshelled under aseptic conditions and 49 nuts were placed onto seven petri dishes containing Malt Extract Agar. A total of 735 of nuts per brand were examined. The petri dishes were incubated at room temperature at 20°C ± 2°C for 7 to 10 days. During this period the Petri dishes were examined daily and fungi developing and sporulating on or around the nuts were isolated and identified immediately. Slow growing and nonsporulating fungi were isolated, subcultured in Malt extract agar and were identified later. Identification was carried out using the keys of Ellis (1971, 1976), Moubasher (1993), Raper and Fennel (1965) and Samson and Pitt (1985).

EXTRACTION OF AFLATOXINS BY ELISA METHOD: A variety of methods are available to extract aflatoxins from seeds and foodstuff but are laborious and time consuming. The Easi-Extract Column (TD110 Oxoid) is a commercially available extraction column. It involves the binding of aflatoxins in a liquid extract to highly specific monoclonal antibodies contained within a small affinity column. The method is simple, high specific, reliable and can be performed much more rapidly than traditional methods (Candlish *et al*, 1988, 1991).

The sample to be tested was pulverized with a mortar and pestle. Ten grams of the ground sample was mixed with 20 ml of acetonitrile/water (3:2) in a 250 ml beaker for 2 minutes. The mixed sample was then centrifuged at approximately 800g [av.] for 10 minutes at room temperature, 2 ml of the extract supernatant was diluted with 46 ml of phosphate buffered saline, the diluted extract was placed in 50 ml syringe barrel from which the plunger was removed, then the plunger was replaced and the extract was pushed through the Easi-Extract Column TD110 that was attached to the syringe barrel. The bound aflatoxins in the column were eluted with 2 ml methanol, the eluent were applied to chromatoplates which were developed in a solvent system of chloroform acetone (9:1). Aflatoxins B_1, B_2, G_1, G_2 (Sigma, England) were used as standards. One sample from each batch of each branch was analyzed for aflatoxins. Each sample was a mixture of five cans (total of 15 samples representing 5 brands, 15 batches and 75 cans) were analyzed. A detection limit of 2 μg/kg was chosen in accordance with Oxoid instruction leaflet (Oxoidi Total Aflatoxin Easi-Extract Column TD 110. Oxoid Ltd.). Aflatoxins were qualified by visually comparing the intensities of fluorescence of the extract spots with that of the standard after suitable dilutions. The concentration of the aflatoxin in the sample (μg/kg) was calculated according to Singh *et al.*, (1991) by the formula

$$S \times Y \times V / W \times Z$$

where

S = Volume of aflatoxin standard, in μl of equivalent intensity to Z μl of sample

Y = Concentration of aflatoxin standard in μg/ml

V = Volume of solvent in μl required to elute the extract

Z = Volume of sample extract, in μl required to give fluorescence intensity comparable to that of 5 μl of the standard

W = Weight of original sample in g, contained in the final extract

TABLE 1

Percentage Frequency of Fungal Contamination in Different Brands of Pistachio Nuts.

FUNGI	BRANDS					
	1	2	3	4	5	% AVERAGE IN ALL BRANDS
A. niger	8.3	4.5	5.8	40.7	14.8	14.8
Penicillium spp.	8.6	27.7	6.7	12.9	11.9	13.6
A. flavus	4.9	15.5	1.1	13.0	14	9.7
A. nidulans	2.3	4.8	0.4	0.22	0.27	1.6
C. cladosporoides	0	0.53	0.27	3.8	0.13	0.95
A. alternata	0.27	0	1.1	0.27	0.13	0.35
A. ochraceus	0.27	0	0.40	0.13	0.40	0.24
U. consortiale	0	0	0.13	0	0.53	0.13

Results and Discussion

The level of fungal contamination in the different brands of pistachio nuts studied is shown in Table 1. *Aspergillus niger* van Tieghem was the predominant species and was isolated from all the brands with an overall average frequency of 14.8% of nuts in the brands. *Penicillium* spp.were prevalent in all brands studied with an overall average frequency of 13.6% of nuts. *A. flavus* was also found in all brands with an overall average frequency of 9.7% of nuts. This is in agreement with earlier findings (Gawad and Zohri, 1993). Jimenez *et al.* (1991) studied 32 samples of pistachio nuts (1600 kernels) in Spain and found that the percentages of infected kernels of pistachio nuts were 9.8% for *A. flavus*, 21.4% for *A. niger*, and 18.6% for *Penicillium* spp. Heperkan and Aran (1994) reported that the predominant flora on stored pistachio nuts were *Aspergillus (A. niger* represented the majority of *Aspergillus* species), *Penicillium* and *Cladosporium*, while Bilgrami and Ghaffar (1994) reported that the predominant species were *A. flavus* and *A. niger*. In the present study, *A. nidulans* was found in all brands but at lower percentage (1.6%) compared with *A. niger* and *A. flavus*. The percentage frequency of *C. cladosporoides*, *A. alternata*, *A. ochraceus* and *U. consortiale* were found to be less than 1% and were absent in some samples.

The number of colonies of *A. flavus* isolates per can sample in the brands shown in Table 2 [was analyzed using a one-way ANOVA test]. The analysis showed a significant difference between the brands ($P<0.05$). The table shows the highest contamination by *A. flavus* in brand 2. The lowest level of contamination is shown by brand 3. Turkey's test showed similarity in contamination between brands 1 and 3 and between brands 2 and 4.

BATCHES OF PISTACHIO NUTS: The companies marketing pistachio nuts release their products in batches at different times. The country of origin of the pistachio nuts were not indicated on the cans. One would assume that if the pistachio nuts are always coming from the same source and are processed under the same conditions, the percentage contamination of the batches by *A. flavus* as well as other fungi would be constant. In this study significant differences were found among all the batches contaminated by *A. flavus* except brand 3 (Table 2). A one-way ANOVA test showed that there was significant difference at the levels of d.f.=14, $F = 3.6$, $P<0.05$; d.f.=14, $F = 15.3$, $P<0.00$;d.f.=14, $F = 14.3$, $P<.00$; and d.f.=14, $F = 4.4$, $P<0.03$ for the batches of the brands 1, 2, 4 and 5 respectively. These significant differences among the batches may indicate differences in the quality of the pistachio nuts as far as *A. flavus* contamination is concerned. Studies on pistachio nuts in Japan (Tabata *et al.*, 1993) have shown that the difference in aflatoxin contamination was related to the country of origin where aflatoxin positive samples were found in samples from Iran where as samples from USA were free of aflatoxin.

ISOLATES AND TOXIGENIC STRAINS: Many species of *Aspergillus* and *Penicillium* are known to produce

TABLE 2

The Number of A. Flavus Isolates in Batches and in Brands.

Brands	Batches	Can 1	Can 2	Can 3	Can 4	Can 5	Anova test
	1	3	3	3	4	3	d.f = 14
	2	2	2	1	6	3	F = 3.6
1	3	2	1	2	0	1	P < 0.05
	1	23	12	10	17	11	d.f = 14
	2	6	4	5	9	15	F = 15.3
2	3	1	1	0	0	0	P < 0.00
	1	2	0	0	0	0	No
	2	0	1	0	2	1	significant
3	3	0	0	0	1	1	difference
	1	2	18	18	27	20	d.f = 14
	2	0	3	1	0	0	F = 14.36
4	3	0	3	0	1	3	P < 0.00
	1	11	14	6	2	10	d.f = 14
	2	13	10	12	0	14	F = 4.4
5	3	4	1	3	1	12	P < 0.03

mycotoxins. In the present study, potentially toxigenic fungi such as *A. flavus* (9.7%), *A. ochraceus (0.4%), A. nidulans* (1.6%), *Penicillium* spp. (13.6%) and *A. alternata* (0.35%) have been found in the different brands studied (Table 1). The presence of these potentially toxigenic moulds in pistachio nuts or any other food product indicate the potential for mycotoxin contamination. If these fungi find suitable conditions for their growth they produce mycotoxins. Although not all *A. flavus* strains are aflatoxigenic, a high incidence of aflatoxigenic strains are usually found among the isolates. In Israel, 1626 isolates of *A. flavus* from groundnuts were examined and 89.6% of them were found to be aflatoxigenic (Joffe, 1969). Lisker *et al.* (1993) surveyed 17 reports collected from works performed all over the world and found that 14 of them showed 50-100% of the isolates to be aflatoxigenic. They also found that 77% of the two hundred strains they isolated from groundnuts were aflatoxigenic. In Australia, 49% of the strains of *A. flavus* in animal feed were found aflatoxigenic (Cannole *et al.*, 1981). In Turkey,35.2% of the isolated *A.flavus* [18/51] were found to be toxigenic (Herperkan *et al.*, 1994).

AFLATOXINS IN PISTACHIO NUTS: Pistachio nuts have been reported to be contaminated by aflatoxins at different ratios and concentrations. In Switzerland Steiner *et al.*, (1992) found that the ratio of 4700 uncontaminated pistachio nuts kernels to one kernel containing aflatoxin B_1 at a level ranging from 8 ppb to 61 ppb. In Japan (Tabata *et al.*, 1993) of 165 samples of pistachio nuts examined, 5 were found containing aflatoxin at an average of 323 ppb for B_1, 58 ppb for B_2. In the present investigation, only one sample out of 15 assayed was found to contain aflatoxin B_1 at a level of 20 ppb. Similar results were reported in which aflatoxins were found in only one sample [13 ppb] of pistachio nuts from 30 samples surveyed (Burdaspal *et al.*, 1990). In the USA aflatoxins were detected in 11 of 17 orchards studied at a level ranging from 0.1 to 958 ppb (Doster and Michaillides, 1994). No aflatoxins were found in pistachio nuts surveyed in Saudi Arabia (Abdel-Gawad and Zohri, 1993) and Turkey (Herperkan *et al.*, 1994).

The presence of aflatoxins or potential mycotoxin producing fungi in pistachio nuts is a potential health hazard. Many countries regulated the maximum permissible levels of aflatoxins in foods, as follows: 20

ppb in the USA, 15 ppb in Canada, 10 ppb in France, UK and Japan, and 5 ppb in Australia (Tabata *et al.*, 1993).

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