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Toxigenic Fungi Isolated from Dried Fruits: Detection of *Aspergillus* Toxins in Saudi Arabia

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Mycotoxins are the toxic secondary metabolites produced by fungi including mushrooms, molds, and yeasts. They are found in many food and food-products, dry-fruits and nuts. These are responsible for infection in animals, plants and human beings (mycotoxicoses). The present study is focused on the detection and management of toxigenic fungi isolated from different dry fruits and nuts. Predominant genera were *Aspergillus niger* (50.8%), *A. flavus* (19.5%) and *Penicillium digitatum* (10.4%). The mycotoxin produced from *Aspergillus* spp. Isolated from different dried fruits using ELISA were identified as B1, B2, G1, G2, Fumagilin and Maltoryzine. *A. flavus* was efficient than *A. niger* in producing G1, G2, B1 and B2. On the other hand, there were non-significant differences between *A. flavus* and *A. niger* in producing fumgallin and Maltoryzine.

Key words: Toxigenic fungi, Dry fruit, *Aspergillus* species, HPLC.

There is a wide range of food is contaminated with mycotoxins, and the contamination occurs throughout the food chain, through the pre- and postharvest periods. Processing steps such as drying, storage and transport play an important role in food safety. Although aflatoxins and ochratoxin (OTA) are the two generality abundant mycotoxins to food contamination, aflatoxins are frequently considered to be field contaminants while OTA is a drying and storage-related mycotoxin produced by *Aspergillus* and *Penicillium* species[1,2,3]. OTA was isolated first as a secondary metabolite of *Aspergillus ochraceus*[4]. Several authors discovered that many other *Aspergillus*[5] and *Penicillium* spp.[6] have the ability to produce OTA when the relative humidity, temperature and product moisture are favorable[7]. The toxigenic species of *Aspergillus carbonarius*, *A. ochraceus* and *Penicillium verrucosum* can grow and produce OTA at low, moderate and high temperatures, respectively, extensive incidence of OTA has been reported in a variety of diverse geographical districts and climates, and in many foods and drinks[2,3].

Furthermore, using HPLC, researchers have discovered that OTA contamination is not merely a problem for improperly stored cereal products, as previously proposed. A diversity of foods, such as bread, wine, coffee beans, grape juice and dried fruits have also been found to be contaminated [8,3,9]. Because of the carry over effect, feeding animals with contaminated crops will also transfer this toxin to animal products consumed by humans. Milk, kidneys and other internal organs

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pose a risk to public health[10,11,12]. There are a lot of evidence for carcinogenic, genotoxic, immunotoxic, teratogenic, nephrotoxic and immunosuppressant activity of OTA in experimental animals[2,13] and OTA is believed to cause urinary tract tumors and Balkan-endemic nephropathy in humans[14]. Furthermore, the International Agency for Research on Cancer (IARC) has classified OTA as a possible human carcinogen (group 2B)[15]. Dried vine fruits are healthy foods and are also ingredients in muesli, cereal bars, biscuits and cakes, among other foods, and could be an important source of OTA for people who consume large amounts, mainly children. The European Union has recently established extreme OTA limit of 10 µg/kg for these products[16]. The aim of our study was to determine the incidence of toxigenic fungi in dried fruits of Saudi Arabia with special interest in OTA producing black aspergilli.

MATERIALS AND METHODS

Isolation, characterization and identification of mycoflora from dried fruit

Mycoflora isolation

The fruits were collected from many markets in different areas in the Kingdom of Saudi Arabia which produce in many different countries. All the dry fruits used in this experiment were apparently free from physical damage and diseases, and make two groups from each fruits. Fungi were isolated on Dichloran 18% Glycerol Agar (DG18)[17] with and without surface disinfection. The dried fruits were disinfected externally by immersion in a 2% sodium hypochlorite solution for 1 min, then rinsed with sterile water. All plates were incubated in darkness for 7 days at 25 ºC. Strains were sub-cultured on potato dextrose agar (PDA) slants, allowed to grow at 25 ºC for 7 days and stored at 5 ºC for final identification to species level. Second group of dried fruits were put on PDA without surface sterilization. Taxonomic identification was made according to Pitt[18], Pitt and Hocking[17], Klich[19] and Samson *et al*.[20]. The incidence of occurrence of each fungus was calculated using a formula[21].

Determination of percentage occurrence of the fungal isolates

This was done to determine the incidence of occurrence of the different fungal isolates. The frequency of occurrence of the pathogens from the dried fruits was determined. The total number of each isolate in all samples was obtained against the total number of all the isolates in all the samples screened. The mean value of this gives the percentage of occurrence as the following equation shows:

$$\% \text{ of occurrence} = \frac{X}{N} \times 100$$

Where X = total number of each isolate in all samples and N = total number of all the isolates in all the samples.

Mycotoxins producing screening test by use UV Light

The Ultra Violet light was used as simple screening test for mycotoxins production. The presence or absence of fluorescence in the agar surrounding the colonies assayed was determined under UV radiation (365 nm) and expressed as positive or negative. The results were photographed using the UV camera. The positive strains were detected for Toxins.

Experiment testing the sensitivity of the okra plant fungal toxins

The isolated fungi were inoculated on PDB to make inoculation of the toxin producing fungi, incubated for 7-15 days at 25±2°C[22]. A filter paper (weighed at the beginning) to filtrate the broth, after that rolled the filter papers containing the filtered toxin producing fungus and then dried it in an electric oven at a temperature of 40°C, until the disposal of any fluid containing in the filter paper (while retaining the filtrate for the usage in infection of okra plant). Reweight of filter paper containing the fungus and then find out the dry weight of fungus. The seeds of okra were planted in small pot until the beginning of a small leafs growth. Some wounds were made in the plants using carborundum to facilitate the induction of the fungi to the plant. Plants were infected by the previous filtrate using the pipettes. The plants were examined for one to two weeks until the symptoms of fungal infection appeared. The fungal growth on plants were examined and photographed.

Quantitative analysis of fungal mycotoxins

Aflatoxin analysis in samples by indirect competitive ELISA

The quantitative analysis of AF in samples was performed by indirect competitive ELISA individually as B1, B2, G1, G2, Fumagilin

and Maltoryzine. The protocol was similar to that for determining antibody specificity except that AF standards concentrations ranging from 0.04 ng/ml to 5 ng/ml were prepared in AF-free samples extract (zero level). Fifty microliter aliquot of each sample was added to a well containing 50 µl of purified antibodies which was diluted 1:8000. The calibration was obtained by plotting of AF standard against optical density at A492. Concentration of AF in the samples extract was determined per milliliter using the following formula: AF concentration (ng/ml) in sample extract sample dilution factor. For the recovery test of AF from spiked samples, AF standards were added in 10 ml samples known not to contain detectable AF to obtain concentrations ranging from 0.01 to 3.2 ng/ml, and then extracted and assayed as described above[23,24].

RESULTS AND DISCUSSION

Effect of sterilize surface and non-sterilize surface in number of isolates from dry fruits

A high level of fungal contamination was detected in most of the samples (Table 1). Although surface disinfection generally reduced the number of dried fruits with viable mold, there was considerable internal mold invasion. The data in Table 1. Showed that the raisins gave the highest total number of fungi isolates which were 24 isolates, while the lowest number of fungi isolates which was 12 were given by strawberry and the plums. There is different in numbers of isolated fungi from sterilized dry fruits and non-sterilized dry fruits, all the time the highest number of fungi isolates were produced from the non-sterilized dry fruits, but the sterilized dry fruits gave the lowest number of fungi, for example non-sterilized raisins isolates were 17 but sterilized raisins isolates were only 7. Dry lemon gave 18 isolates, 7 isolates from sterilized lemon while 11 isolates from non-sterilized, on the other hand there are no significant different between sterilized and non-sterilized dry fruits such as Apricot, strawberry and cherry were 7-7, 6-6 and 6-7 in cherry. In some dry fruits the number of fungi isolates which isolated from the surface of sterilized dry fruits were higher than fungi isolates from non - sterilized surface dry fruits for example sterilized fig gave 8 isolates while non-sterilize fig gave only 3 isolates. Sterilized pineapple (ananas) gave 11 isolates in the non-sterilized pineapple the isolates were only 3.

Percentage of fungal profiles isolated from dry fruits

The isolated fungi from dry fruits were *A.niger*, *Macrophomina phaseolina*, *Alternaria alternata*, *PeniciIlium digitatum*, *P. italicum*, *Aspergillus terreus*, *Fusarium chlamydosporum*, *Aspergillus wenti*, *Cadosporium herbarum* and *Aspergillius flavus*Grape (raisins) yielded the highest number of fungi[6] while dried Mango lowest yielded the lowest number (1) (Table 2). *A.niger* showed the highest isolation frequency (50.8 %) and *A. flavus* isolation frequency was (19.5%), while there were no significant differences in isolation frequency between the other fungi which showed isolation frequencies ranged from 0.3 to 10.4 %. (Table 3.). In previous studies, percentages range from 0.6 to 18.5% [25,26,2728]. Nevertheless, Da Rocha Rosa *et al*.,[29] stated that 30% of *A. niger* from grapes in Brazil was mycotoxin producers, while Serra *et al*.,[28] found an unusually high percentage (43.1%) of ochratoxigenic isolates in the species *A. niger* var. *niger* from grapes in Argentina. A high proportion (96%) of *A. flavus* and *A. niger* screened in the present work produced mycotoxins[26]. The consistent ability of this species to produce OTA has also been reported by other authors [29,27,30,28). Toumas and Katsoudas[31] found that berries had the highest levels of contamination; strawberries (97%

Table 1. Isolation fungi from sterilized and non-sterilized dried fruit

Fruit	Sterilized	non-sterilized	Total isolate
Grape	7	17	24
Lemon	7	11	18
Coconut	9	8	17
Date	9	7	16
Fig	8	3	11
Mango	5	6	11
Apricot	7	7	14
Papaya	5	8	13
Banana	6	9	15
Prune (plums)	4	8	12
pineapple	11	3	14
Strawberry	6	6	12
Cherry	6	7	13
Kiwifruit	2	11	13
pomegranate	9	7	16

Table 2. Percentage of fungal profiles isolated from dry fruits

Fruit	*A. alternata*	*A. niger*	*M. phaseolina*	*P.italicum*	*P. digitatum*	*C. herbarum*	*F.chlamydosporum*	*A.flavus*	*A. terrus*	*A. wentii*
Grape	8.3	66.7	4.2	4.2	0.0	0.0	8.3	4.2	4.2	0.0
Lemon	0.0	50.0	0.0	0.0	12.5	0.0	0.0	37.5	0.0	0.0
Coconut	0.0	42.9	0.0	0.0	0.0	0.0	0.0	57.1	0.0	0.0
Date	16.7	66.7	0.0	0.0	0.0	0.0	0.0	16.7	0.0	0.0
Fig	0.0	45.4	0.0	9.1	9.1	9.1	9.1	18.2	0.0	0.0
Mango	0.0	100	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Apricot	0.0	50.0	0.0	0.0	0.0	0.0	25.0	25.0	0.0	0.0
Papaya	0.0	0.0	0.0	33.3	0.0	66.7	0.0	0.0	0.0	0.0
Banana	0.0	40.0	0.0	0.0	20.0	0.0	0.0	20.0	0.0	20.0
Prune	0.0	100.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Pineapple	0.0	25.0	0.0	0.0	0.0	0.0	0.0	75.0	0.0	0.0
Strawberry	0.0	50.0	0.0	0.0	0.0	0.0	0.0	50.0	0.0	0.0
Cherry	0.0	66.7	0.0	0.0	0.0	0.0	33.3	0.0	0.0	0.0

Table 3. Frequency of fungal isolated from dry rot

Fungus	Frequency (%)[a]
A. niger	50.8[a]
A. alternate	2.5[c]
P. digiatum	10.4[c]
P. italicum	2.8[c]
M. phseolina	0.3[c]
A.wentii	1.8[c]
A.terrrus	2.2[c]
A. flavus	19.5[b]
F. chlamydosporum	4.6[c]
C. herbarum	5.6[c]

A each value is the mean of 15 replicates (fruits) percentage data were transformed into $\sqrt{\times}+0.5$ before carrying out ANOVA to produce approximately constant variance. Mean's followed by the same letters are not significantly different (P<5%) according to Duncan's multiple range test.

Table 4. Testing the sensitivity of the okra plant fungal toxins

Dry fruit	Fungus	Infection okra plant
Date	*A. flavus*1	++
Date	*A. flavus*2	+++
Lemon	*A. flavus*3	-
Date	*A. flavus*4	+
Strawberry	*A. flavus*5	+++
Plums	*A. flavus*6	+
Coconut	*A. flavus*7	++
Pineapple	*A. flavus*8	-
Banana	*A.wentii*	+
Prune	*A. niger*1	+++
Date	*A. niger*2	+++
Fig	*A. niger*3	++
Kiwifruit	*A. niger*4	+++
Date	*A. niger*5	+++
Date	*A. niger*6	++
Grape	*A. niger*7	+
Mango	*A. niger*8	+++
Grape	*A. niger*9	++
Lemon	*A. niger*10	++
Cherry	*A. niger*11	-
Strawberry	*A. niger*12	++
Pineapple	*A. niger*13	+++
Coconut	*A. niger*14	++
Apricot	*A. niger*15	+
Date	*A. niger*16	+
Grape	*F.chlamydosporum*1	-
Grape	*F.chlamydosporum*2	+
Fig	*F. chlamydosporum*3	-
Grape	*P.italicum*	++
Fig	*P.digitatum*1	+++
Pomegranate	*P.digitatum* 2	++
Date	*A.alternata*1	+
Grape	*A.alternata*2	+

infected) among fruits which were the most susceptible probably due to the fact that their skins are soft, easily ruptured with numerous indentation and hair-like protuberances which allow most organisms to attach and proliferate[31].

Mycotoxins producin screening test by UV Light

The UV light for the screening of all the fungi to identify which isolates produce mycotxoin, the isolates which produce mycotoxin gave blue green light around the mycelium on the plate (A) while it was dark and did not produce any light around the isolated fungi which do not produce mycotoxin (B). This findings supported by the work of Sekar *et al.*,[32] in India, they proved the efficacy of the aflatoxin-screening processes by UV-light.

Testing the sensitivity of the okra plant for fungal toxins

Data in Table 4 showed that sensitivity of okra plant to produce mycotoxin by isolated fungi from dried fruits, this test was used for the screening of fungi produce mycotoxins, some fungi can produce mycotxoin in liquid media, when spread or injected in okra plant which sensitive mycotoxin it make some symptoms on the okra leaves such as change the green color to yellow color or brown and kill the plant. The results record as (+) mean isolate fungi produce mycotoxin (-) means that fungi not produce mycotoxins. *A. flavus*2 which isolated form date, *A. flavus*5 isolated from s strawberry and *A.niger* 1,2,4,5 and 8 gave highly positives effect in okra leaves +++. The leaves changed into yellow and brown colour , while *A. flavus*3 which isolated from lemon, *A. flavus* 8 from dry ananas, *A. niger*11 from cherry and *F. chlamydosporum* l,3 isolated from raisins and fig gave negative effect - in okra plant, the leaves still green (Table 7). These results are in agreement to some studies performed in many countries and different types of fruits[33,34,35].

Quantitative analysis of fungal mycotoxins

Determination of mycotoxins production by *Aspergillus* spp. Using indirect competitive

Table 5. Determine mycotoxins produce by *Aspergillus* spp. by indirect competitive ELISA

Dry fruit	Isolate	Mycotoxins (µg)					
		B1	B2	G1	G2	Fumagilin	Maltoryzine
Grape	*A. flavus*	7.75	6.2	9.61	4.03	0.0	4.4
Date	*A. flavus*	6.2	3.4	2.3	2.7	0.0	2.1
Date	*A. flavus*	4.1	4.7	0.0	0.0	0.1	1.9
Lemon	*A. flavus*	6.8	11.4	5.2	7.4	0.0	0.0
Date	*A. flavus*	3.5	5.3	8.9	2.7	0.0	3.3
Strawberry	*A. flavus*	4.9	8.9	9.6	10.2	0.0	0.9
Plums	*A. flavus*	10.2	4.3	3.5	2.3	0.0	3.8
Coconut	*A. flavus*	1.9	2.2	3.1	0.6	0.4	3.0
Date	*A.niger*	-	-	-	-	-	-
Banana	*A.wentii*	0.8	0.2	0.1	0.0	11.2	2.0
Prune	*A.niger*	0.6	0.4	0.2	0.1	9.9	0.7
Date	*A.niger*	0.1	0.2	0.0	0.1	0.0	4.2
Fig	*A.niger*	0.2	0.1	0.4	0.0	0.0	3.3
Kiwifruit	*A.niger*	0.0	0.0	0.0	0.0	0.0	1.4
Date	*A.niger*	0.1	0.4	0.0	0.2	0.2	0.9
Date	*A.niger*	0.2	0.1	0.0	0.0	0.0	1.7
Grape	*A.niger*	0.3	0.0	0.0	0.1	0.3	3.2
Mango	*A.niger*	0.2	0.0	0.2	0.2	0.0	0.4
Grape	*A.niger*	0.1	1.0	0.0	0.0	0.4	2.6
Lemon	*A.niger*	0.1	0.0	0.1	0.2	0.1	4.1
Cherry	*A.niger*	0.0	0.0	0.0	0.0	0.2	3.8
Strawberry	*A.niger*	0.0	0.0	0.0	0.0	0.0	5.1
Pineapple	*A.niger*	0.4	0.2	0.3	0.0	0.0	6.3
Coconut	*A.niger*	0.6	0.1	0.2	0.1	0.2	0.9
Apricot	*A.niger*	0.1	0.1	0.0	0.3	0.0	4.6
Pineapple	*A.flavus*	0.1	0.1	0.2	0.1	0.1	3.7

ELISA, Which isolated from dry fruits. The mycotoxins B1, B2, G1, G2, Fumagilin and Maltoryzine produced from26 isolates *Aspergillus* spp.

The data in table 8 showed that the isolate of *A. niger* which isolated from date did not produce any kind of toxins on opposite of *A. flavus* from coconut which yield all the 6 toxins by little amount of toxins compeer the other. Toxin Maltoryzine was produced by completely all isolates of Aspergillus spp except for one isolate, the range was between 0.4 - 6.3 µg, *A. flavus* which isolated from raisins gave a highly concentration of mycotoxin Bl,B2, G1, G2 and Maltoryzine receptively 7.75, 6.2, 9.61, 4.03 and 4.4 and so two isolates of *A.flavus* which isolated from date and dry lemon gave highly concentration of mycotoxin B1,B2, G1, G2 and Maltoryzin too. The data in table 5 explain the most of isolates of table can produce two or three mycotoxins at last. *A.niger* from dry strawberry produce only one toxin. The same findings obtained by other study[33], but Ahmed *et al*.,[36] found significant mycotoxins production during Khalal stage of dates. These differences may be because they focus on this stage of dates or because their date fruits suffered mechanical damage in the field or during harvesting. Also it was noticed that the toxin Maltoryzine was produced by completely all isolates of *Aspergillus* spp. Except for one isolate. *A. flavus* which isolated from raisins gave a highly concentration of mycotoxin B1, B2, G1, G2 and Maltoryzine while other study found only B1[37] may be due to their limited methods and samples. Also two isolates of *A. flavus* which remote from date and dry lemon gave highly concentration of mycotoxin B1, B2, G1, G2 and Maltoryzin too. Also, it was found that the toxin B 1 produced by *A. niger* was positively correlated with each of G1 (P<0.05) and Fumgillin was positively correlated with each of B1 and B2 (P<0.05), while all the other correlations were non-significant.

When study Correlation among in toxins production by isolates of *A.niger*

Data in Table 6. showed that B1 was positively correlation with each of G1 (P<0.05) and Fumgillin was positively correlated with each of B1 (P<0.05) and B2(P<0.05). All the other correlations were non-significant. Data in table 10 showed that Correlation among in toxins production by isolates of *A.flavus*. G1 was positively correlated with each of B2 (p<O.05) and G 1 (P<0.05). All the correlations were non-significant.

Efficiency of *A. flavus* and *A. niger*in producing mycotoxins in dry fruits

Data in Table 5. showed that *A.flavaus* was efficient than *A.niger* in producing G1 , G2, B1 and B2. Also, the differences between *A. flavus* and *A. niger* in producing fumgallin and Maltoryzine were non-significant. Our results showed that correlation in toxins produced by isolates of *A. flavus*. G 1 was positively correlated with each of B2 and G1 (P<0.05). Member of the *A. flavus* group are widespread in the most part of the world and they are capable of growing on, and producing aflatoxins in, a wide range of foods and feeds. However, not all strains of *A. flavus* produce aflatoxins[38].

Black-sporedaspergilli are difficult to classify and the taxonomy of this section is still unclear[39]. Traditionally, the classification of this section was based on morphological characteristics. However, recognition of phenotypic characters within this group by an inexperienced diagnostician is a tremendous challenge. A diagnostic phenotypic procedure based on biochemical traits on agar media along with some molecular approaches has been recently reviewed [40].

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