Detection of Fungi Contaminated some Nuts and Its Ability for Aflatoxin B1 Production

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Abstract. This study was conducted in February and March 2021 to collect data on the incidence of aflatoxinB1 (AFB1) and the ability of fungi isolated from nuts (almond, pistachio, hazelnut, cashew, imported and local peanut) to produce AFB1 in Ramadi city, Iraq . The mycological investigation revealed the isolation of fourteen fungal species from eight genera, *Aspergillus flavus* was the most common and widely distributed fungus. TLC analysis was used to assess *Aspergillus flavus'* ability to produce aflatoxinB1. The majority of *A.flavus* isolates In the culture media(YES) were toxigenic and capable of producing aflatoxinB1 . The ability to produce aflatoxin B1 ranged from 30 - 40 ,25 – 60 , 15 – 50 , 20 – 20 , 40 – 70 , 65 – 150 ppb in almond , pistachio , hazelnut , cashew , imported and local peanut respectively . The ELISA approach was used to estimate the natural occurrence of AflatoxinB1 in nuts samples . Contaminated samples with aflatoxinB1 were 40,20 , 40 , 40 , 80 and 80 % of tested samples for almond , pistachio , hazelnut , cashew , imported and local peanut , Concentrations of aflatoxin B1 were ranged between 9 – 16 , 17 – 45 , 20 – 26 , 10 – 12 , 180 – 190 , 150 – 225 ppb respectively . The results show that most samples have a high contamination of AlatoxinB1 that is higher than health standards for consumption (20ppb). Mycotoxigenic *A. flavus* isolates are high contaminants for food and should be removed to reduce the risk of nut toxin contamination.

Keywords. Nuts, Toxigenic, Fungi, Aflatoxin, Contamination, Ramadi.

1. Introduction

Nuts have high nutritional value for each roasted or fresh consuming . It is very richness of protein , fat , carbohydrate , amino acids and vitamins. Almost all types of nuts are utilized in a variety of industries, including pastries, sweets, cosmetics, resins, ets, and direct consumption. Because its highly content of proteins , fat and low water , various nuts such as almond (Prunus dulcis) , pistachio (Pistacia vera L) ,hazelnut (Corylus avellana) , cashew (Anacardium occidentale L) , peanut (Arachis hypogaea) are vulnerable to be attack by pre and / or post- harvest molds [1-3] . Mold can grow rapidly upon nuts if there were inappropriate marketing and storage. Contaminate of fungus for various types of nuts may occur at three stages, it may occur first when the nuts are on the tree, so that they are frequently attacked by spores of several fungus species that are carried in the air or could be contaminated with fungus through de-hulled and washed after harvesting as a second stage. Most commonly the last stage can occur during storage, when nuts are not sufficiently dried or are stored under severe temperature and humidity conditions . Over thousands fungal species are currently classified as natural pollutants of agricultural and food items, including toxigenic and pathogenic fungal species [4]. Mycotoxins are toxic secondary metabolites produced by various types of fungus

that can contaminate crops , affecting public health and the agricultural economy[5] These mycotoxins have a significant and negative influential role on health , their effects vary according to their types and concentration of toxin in polluted material , in addition to other environmental and vital factors, which affects the performance of the exposed. The risk of mycotoxins is increasing in the event of more than one kind of toxins in the same agricultural or food commodity. A lot of fungal species have ability to produce more than one kind of toxins, also many different fungal species have the ability to secrete the same kind of mycotoxin. current data has revealed the presence of several mycotoxins in food samples, which has major implications for both food safety and human health. So far, more than 3 hundreds of mycotoxins have been found, with roughly a dozen garnering the most attention due to their severe impacts on human health and presence in food. Aflatoxins (AFs) are thought to be the most carcinogenic compound naturally produced than other mycotoxins. *Aspergillus flavus, Aspergillus parasiticu, Aspergillus nomius ,* and other species are toxigenic molds , can capable of flourishing in such these kind of nuts (almond, cashewnut, hazelnut, Peanut *etc*.) . The Rapid Alert System for Food and Feed (RASFF) reported 669 alerts or notifications for mycotoxins in 2009, 95 percent of which were for AFs, mostly from nuts [6]. All types of nuts imported from outside Iraq except peanut , which are implanted in Anbar province as a major crop . The present study was aimed to Detection fungi contaminated some imported and local nuts such as almond, cashewnut, hazelnut, Peanut and pistachio(Tab.1) and its ability for aflatoxin B1 production in the markets of Ramadi city , Anbar province , Iraq .

2. Material and Methods

2.1. Sampling

Sampling was done for five kinds of imported nuts included pistachio , almond , cashewnnut , hazelnut , local and imported peanut (Tab.1). Nuts samples were collected randomly from 10 different markets location , with each sample obtained from a different vendor in Ramadi city during the months of March and April 2021, to be used for fungal detection , isolation and determination of natural occurrence AFB1.

Table 1. Scientific and English names of the studied nuts.

No	Scientific name	English name
1	*Prunus dulcis*	Almond
2	*Anacardium occidentale*	Cashews
3	*Corylus colurna*	Hazelnut
4	*Pistacia vera*	Pistachio
5	*Arachis hypogaea*	Peanut(imported)
6	*Arachis hypogaea*	Peanut(local)

2.2. Isolated Fungi Found with Nuts

Isolated of fungi that present in nuts were followed, Depending on the method by [2]. Each sample was divided into two parts , the nuts were sterilized surface in first part and the other was not . First part samples were sterilized surface in a flask with a 6 percent sodium hypochlorite solution (NaOCl) for 2-3 minutes before being washed with distill water for 2 minutes. Fungi were isolated by randomly selecting 100 grains of two each part from each location and directly plating them in Petri-dishes at a rate of (4-6) pieces per plate, (nuts samples of large size cut into smaller particles) on Czapek's Dox agar medium (CDA).The samples in Petri-dishes were then kept at 25 ± 2^0C for 7 days. The percentage of infected grains in the samples was calculated , then the grown colonies of fungus were counted , isolated and kept after purified on Petri- dishes potato dextrose agar (PDA) medium for diagnosis .

2.3. Isolation of Sample Surface Mycoflora

Sterile forceps were used to transfer the samples into the Petri dish, which contained sterilized CDA. Three duplicates of each type of nut were performed. and the plates were kept in incubator at 25±2°C for 7 days . After incubation ,the fungi colonies were diagnosed according to their morphological and

microscopic features. Based on its taxonomic keys ,fungus were identified by used texts(books) : [7-9].

2.4. Ability of A. flavus isolates for AFB1 Production :
According to [10], the ability of *A. flavus* strains to produce AFB1 in liquid media Yeast Extract Agar(YES) was investigated. Quality detection for AFB1 were done for all (42) isolates have been obtained by Thin Layer Chromotography (TLC) technique .

2.5. Standard work solution:
Aflatoxin standards were prepared in accordance with the Association of Official Analytical Chemists, A.O.A.C. [3]. Aflatoxin B1 crystals were diluted in benzene-acetonitrile (98:2 v/v) to a concentration of 10 µg/ml (stock solution).

2.6. Determination of natural AFB1 in samples using ELISA technique
Three samples of each kind were randomly selected for quantity detection of aflatoxinB1 . Enzyme Linked Immunosorbent Assay technique (ELISA) was used. The aflatoxin assay was performed according to the instructions provided by the manufacture (Sheuzhes Lvshiyuan Biotechnology co., Ltd . Samples (5 g) were putted in 25 mL flask with 70% of methanol for aflatoxin analysis. The sample was shacked vigorously for five seconds by vortex several times. 50 µl of each sample and stander solution of aflatoxin B1 put in well plat by multi micro syringe. The plat was covered with tape and incubated for 30 minutes at room temperature . well plate were emptied and washed with 500µl of buffer provided with assay kit ,washing process was repeated 5 times then the plat dried . 50 µl of both substrate A and B were poured in each well plat and incubated for 20 – 30 minutes at room temperature Finally to stop the reaction, 50 µl of stopping solution was added. The absorption was detected by ELISA reader (Biotick) at 450nm wave length . Aflatoxin in samples was calculated from the standard curve derived from aflatoxin standards and expressed in part per billion (ppb).

3. Results

3.1. Isolation and identification fungus
Many fungi are known to grow on/in nuts, many of these fungi cause deterioration and loses marketing value in stored nuts. As a result, the current study began with the isolation and identification of such fungal species contaminated some imported and local nuts from different markets location in Ramadi city . Fungi were isolated using the Agar Plate Method (APM) plated on CDA medium, and two sets of samples were analyzed, namely, surface sterilized and unsterilized nut samples, as shown in Table 2. The results of tested samples of nuts from domestic markets of Ramadi city showed a number of associated fungi. Eight different fungal genera and fourteen different species were isolated. The fungi were identified using cultural and morphological characteristics,. These were found to be *Aspergillus nige, A.flavus , A. ochracious , Penicillium citrinum , Penicillium chrysogenum ,Penicillium spp , Rhizopus stolonifer , Fhizopus spp , Cladospoium spp , Mucor spp ,Fusarium oxysporum , Fusarium spp , Trichoderma spp , Alternaria alternate* . The Isolates of *Aspergillus* spp were the most appearance in samples and the presence of *Aspergillus* spp was almost repeated in all samples under study.
The data revealed that the surface Sterilized samples (with sodium hypochlorite) produced a lower population of sample-borne fungi than the unsterilized surface. The most prevalent and widely distributed species were *A. flavus and A . niger* across samples of The isolated fungi, while *Alternaria alternate* and *Trichoderma* spp were the least. The data showed that nuts samples collected from retail markets of Ramadi city was infected of fungi with100%. The average of percentage for fungal genera in infected nuts were 53, 44, , 2 and > 1% others in almond, 57, 33 , 9 and > 1% others in Pistachio, 51, 36 , 12 and > o.1% others in hazelnut, 50,40 and 10% in Cashew,57, 33 10% in imported peanut, 65 , 24 , 8 and 3% others in local peanut, respectively. The increase of infection in local peanut is more than other nuts probably due to poor agricultural management in local fields as well as bad drying , handling and storage . The findings are consistent with many researcher data on seed diseases

According to [11], the most commonly present species in infected nuts in Turkey were Aspergillus spp.(1) referred that the most frequent and distributed fungus was *Aspergillus niger* in cashew in Saudi arabia. A total of 60 analyzed nuts samples showed that Aspergillus spp, Penicillium spp, Mucor spp and Fusarium spp were the most isolated fungus from different infected nuts . Some strains of these fungus are potential danger and produce toxic metabolites [12]. However; Mycotoxigenic Aspergillus spp., Penicillium spp., and Fusarium spp. are able to produce various mycotoxins and represent very dangerous fungal genera that infect or contaminate nuts.

Table 2. Isolates of fungi obtained from sterilized and un sterilized nuts on (CDA).

No	Sample name	Czapex dox agar	
		Sterilized surface	**Unsterilized surface**
1	Almond	*Penicillium chrysogenum* *Aspergillus niger* *Aspergillus flavus* *Penicillium spp*	*Rhizopus stonlonifer* *Penicillium spp* *Mucor spp* *Aspergillus flavus* *niger*
2	Pistachio	*Aspergillus flavus*	*Rhizopus spp* *Mucor spp*
3	Hazelnut	*Aspergillus flavus* *A .niger* *Penicillium citrinum*	*Aspergillus niger* *Aspergillus flavus* *Rhizopus stonlonifer*
4	Cashew	*Aspergillus niger* *Cladosporium spp*	*Penicillium crustosum* *Mucor spp* *Fusarium spp* *Alternaria alternate*
5	Peanut	*Aspergillus niger* *Asprgillus flavus* *Penicillium spp*	*Aspergillus niger* *Asprgillus flavus* *Penicillium spp* *Rhizopus stonlonifer* *Fusarium spp* *Aspergillus ochraceous*
6	Peanut (Local)	*Aspergillus niger* *Aspergillus flavus* *Fusarium oxysporum* *Penicillium citrinum*	*Aspergillus niger* *Aspergillus flavus* *Fusarium spp* *Penicillium citrinum* *Rhizopus stonlonifer* *Mucor spp* *Tichoderma spp*

3.2. Occurrence of mycotoxic fungi isolated from nuts

Some kinds of fungus grow without producing mycotoxin and others produce a dangerous metabolites as mycotoxin. The most dangerous adaptations produced for toxins are the fungus dating back to the Aspergillus spp , Penicillium spp and Fusarium spp. The most dangerous fungal species for mycotoxin producing is the type of *A. flavus* belong to Aspergillus spp. As a result, this work was carried out to detect mycotoxic fungus, specifically *A. flavus*, which produces aflatoxin B1. According to the data of table (2), *A. flavus* was the most mycotoxigenic Aspergillus spp, represented by, 80 , 43 , 71 , 52 , 85 , and 82% of Aspergillus spp isolates from almonds, pistachio , hazelnuts, cashews, imported , and local peanuts obtained from markets of Ramadi city . As for AFB1 analysis, the majority of *A. flavus*

isolates were toxic and have ability of generating AFB1 on (YES) medium (table 3) . Data in Table (3) showed that , percentage of *A. flavus* isolates producing toxin were 80 , 42.8 , 66.6 , 100 , 72.7 and 57.1 in almond , pistachio , hazelnuts , cashews , imported and local peanut respectively . Data of TLC technique showed measurable brightness with quantitative concentration ranged between 30-40 , 25-60 , 15-50 , 20- 20 , 40 -70 and 65-150 ppb in almonds, pistachio , hazelnuts, cashew, imported , and local peanuts respectively . Although some isolates did not produce detectable aflatoxins , this may be due to lack of this technique (TLC) to measure low concentrations or to non-genetic ability of isolates to produce Aflatoxin . Also some of the grown isolates were produced aflatoxin B1 and B2 with very low levels of G1 . These results show how dangerous these molds producing toxins on consumer health . The findings are consistent with the findings of many other researchers working on nuts. [13], revered that 16.1% of tested samples were contaminated with AFs and median values of total AFs and AFB1 were 16.6ppb and 15.1ppb, respectively. [14], found Four isolates of the total six isolates of *A. Flavus* have a AFB1 production on the liquid (YES) with concentrate can be detected by TLC technique. In China, [15], investigated the presence of aflatoxin in 370 rice samples collected from six different regions of the country, Of these samples, 63.5% were aflatoxin B1 positive, of which 1.4% contained levels beyond the EU regulatory limits.

Table 3. Number of *Aspergillus flavus* isolates from nuts and its potential to produce AFB1 in YES medium.

type of Nuts	Tested isolates of *A. flavus* (No.)	Positive Isolates for producing AFB1 (No.)	Percentage of *A.flavus* isolates producing AFB1 (%)	Concentration of AFB1 ppb	
				Min	Max
Almonds	5	4	80	30	40
Pistachio	7	3	42.8	25	60
Hazelnuts	3	2	66.6	15	50
Cashews	2	2	100	20	20
Peanut	11	8	72.7	40	70
Peanut (local)	14	8	57.1	65	150

The most prevalent mycotoxin found as a natural contamination in the preserved samples was AFB1.This study was conducted to detect and quantify AFB1 and contamination levels in nuts samples taken from Ramadi city retail markets. The findings of the estimation of the natural occurrence for AFB1 in five nuts samples are shown in Table 4. By ELISA method the concentration of AFB1 were ranged from 9 – 16 , 17 – 45 , 20 – 26 , 10 – 12 , 180 – 190 , 150 – 225 ppb in almond , pistachio , hazelnuts , cashews , peanut , local peanut respectively . The results demonstrate a high AFB1 concentration in most samples, which is more than the normal range for mycotoxin (20 ppb) for human consumption. Aflatoxin levels are highest in peanuts and lowest in almonds and cashews.

Table 4. Concentration of aflatoxin B1 in nuts collected from domestic markets of Ramadi city during Feb. & March 2021.

Type of nuts	No of tested samples	No of positive samples	% of positive samples	AFB1 concentration ppb	
				Min	Max
Almonds	5	2	40	9	16
Pistachio	5	1	20	17	45
Hazelnuts	5	2	40	20	26
Cashews	5	2	40	10	12
Peanut	5	4	80	180	190
Peanut (local)	5	4	80	150	225

Conclusion

The fungi that were detected and diagnosed with high appearance were Aspergillus spp , Penicillium spp and Fusarium spp, In addition to other kinds. These genera are pollutants for many crops, agricultural products and food including nuts. It is a danger to consumers as a fungus for mycotoxins excretion , with low molecular weight . mycotoxins possess high biological activity with a severe effect on public health even in low concentration . AFB1 which excrete from Aspergillus flavus , A. paraciticus and A. nomius can grow in a wild thermal extent that is more dangerous than other toxins and should be controlled**.**

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