Aflatoxins and Aflatoxigenic Fungi Contamination of Dried Fruits in Iraqi Market

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Abstract

Objectives: Studying the incidence of Aspergillus sp. and Aflatoxins (AFs) contamination in dried fruits collected from local market of Hilla city and other origins. Methods: Intergenic fungi were isolated by direct plate method and were morphologically identified on specified media. AFs production by Aspergillus spp. were screened and determined by thin layer chromatography (TLC) and high performance liquid chromatography (HPLC).

Results: Aspergillus spp. represented 93.33% of the total isolated mold species. A. niger was dominant (79.61%) and A. flavus was only 12.5%. Of the total Aspergillus spp., 54.4% found active aflatoxin B1 (AFB1) and aflatoxin B2 (AFB2) producers among 85.24% belong to A. niger and 14.75% belong to A. flavus. HPLC shows that 100% of dried fruit samples tested positive for AFs.

Conclusion: Through our study the mold isolates showed different AFs production behavior that they are able to produce AFB1 and AFB2 at a pH range (3.1 to 4.5), optimum temperature at range (28-32ºC) and low aw for best AFs production and this needs much concern especially with dried fruit storage and treatment conditions.

Keywords: Dried fruit, Aflatoxin, Aflatoxigenic fungi, TLC, HPLC.

Introduction

Dried fruits are vulnerable to fungal and myco toxin contamination for their favorable humidity, high sugar content and other nutrients. Mold infection in dried fruits may occur on the tree through ripening stages, after falling from the tree, through drying process [1] and during storage or refrigeration where Aspergillus niger, found the most abundant (14.92%) followed by Aspergillus flavus (10.14%) in refrigerators [2].

The Mediterranean regions are very favorable for dried fruits production. Now days, dried fruits consumption is wide spread. Almost half of the dried fruits sold all over the world are raisins, Date, and Prune, Fig, Apricot, Peach, Apple and Pears. Dried fruits can support fungal growth, some of which produce mycotoxins. Occurrence of toxigenic molds and mycotoxins on these dried fruits can cause serious health and economic problem in the other parts of the world. The most important mycotoxins occurring in the Mediterranean crops are AFs and OTA where the toxin type, incidence rate of mycotoxins and toxigenic molds vary by crop, country and geographic location [3].

All strains tested from A. flavus and A. parasiticus showed aflatoxigenic potential. A. flavus isolates obtained from apricot and Fig showed the highest abilities (334 and 333ppb), respectively compared with two isolates obtained from dried vine fruits 10.2 and 79.4ppb, respectively and an isolate from plum (37.4ppb). Two isolates of A. parasiticus from Fig showed a marked variation in their AF potential (81 and 344 ppb) [4].

All samples from the crops or dried fruit samples from Iraqi markets were contaminated with mold species. A. niger, A. flavus, A. parasiticus and F. oxysporium a dominant molds when compared with other species.
AFB1 only in high concentration ranged from 38.5-480µg/kg in Fig samples while, the ranges 161-782µg/kg and 22.0-93.7µg/kg in peanut and dried apricot samples, respectively however, other AFs types (AFB2, AFG1 and AFG2) were not detected in all samples [5].

In a previous study concerning Date fruit, Aspergillus spp. represented 84% of the total isolated mold species. A. niger was dominant (65%) and A. flavus was only 19%. Of the total A. spp., 16.6% found active in AFB1 and AFB2 production among of which 45.45% belong to A. niger and 54.54% belong to A. flavus. HPLC shows that 88.8 % of Date fruit samples tested positive for aflatoxins AFs. AFB1 and AFB2 were detected produced by A. niger isolates by TLC. This was the first report that AFG is produced by both A. niger and A. flavus as detected by HPLC. The results showed that Date fruits are contaminated with total AFs ranged from 5.7 to 274µg/kg [6]. The AFs concentration ranges exceeded “at least with some samples” the allowed levels in food materials in Iraq or other countries standard that is 20µg/kg in food samples.

Total of 30 apricot and 15 prune fruit samples were analyzed to evaluate the concentration of AFs by HPLC AFB1, AFB2, and AFG1 were detected in 7 apricot samples. AFB1 and AFG1were detected in 2 prune samples. AFG2 was not detected in any of the apricot and prunes sample. All contaminated samples had a level of total AFs and AFB1 below the Iranian National Standard No.5925. (5ng/g for AFB1 and 15ng/g for total AFs) Therefore, they have no serious problem for the public health. However, it is necessary to monitor the food quality routinely. The results proved the suitability and the effectiveness the immune affinity column clean-up techniques for my co toxins level determination [7, 8].

Materials and Methods
Sample Collection
Fruit samples which include (6) dried fruits (Apricot (Prunus armeniaca), Fig (Ficus carica), plum (Prunus domestica) and Jujube (Ziziphus jujuba)) were collected from local market of Hilla city of different sources and origins. Water activity (aw) and pH of the samples were measured and as the following: aw was calculated by measuring the Relative Humidity using Dual Moisture Meter (Extech Instruments, USA), and divided by 100 as the following formula:

\[
aw = \frac{RH}{100}
\]

The pH was measured by mixing 10g of dried fruit sample with 90ml distilled water (dH₂O) (1/10 sample/water) then homogenized in a blender. The pH value measurements were carried out using pH meter (Extech Instruments, USA) [9].

Samples were collected in sterilized sealed containers then transferred to the laboratory and refrigerated until use.

Fungal Isolation and Identification
Dried fruits taken randomly in triplicates and they were surface-sterilized with 1% sodium hypochlorite solution for 3min [10] then washed by sterile dH₂O and dried on filter paper under aseptic conditions. Samples were cut in small pieces then plated on PDA. All plates were incubated in dark at 25-30°C for 7 days [11]. After the incubation period, total fungal colonies were counted and those belonging to Aspergillus genus were transferred to new PDA plates and incubated at 25-30°C for 5 days in order to obtain pure isolates.

For the identification of Aspergillus spp., pure colonies were grown on two media in triplicates. The media used were as follows: CYA and MEA each medium was supplemented with 250mg/L chloramphenicol. Thereafter, plates were inoculated at three points according to the standardized procedure and incubated in dark at 37°C and 25°C for CYA and at 25°C for MEA, for 7 days [12]. Aspergillus spp. were identified by colony morphology on CYA and microscopically using lacto phenol cotton blue stain through its conidiophores characteristic features, as described by the keys provided by [13-19].

Detection of Aspergillus Toxigenic Strains
The isolates of Aspergillus sp. were screened for the production of the AFs using SMKY liquid medium (sucrose 200g; potassium nitrate KNO₃, 0.3g; magnesium sulfate MgSO₄, 7H₂O, 0.5g and yeast extract, 7g in 1L of dH₂O) [20]. About 1ml of the mold spore
The suspension was inoculated in 25ml of SMKY medium in sterile cups and incubated at 28 ± 2°C for 10 days in triplicates, for AFs estimation [21].

**Toxins Extraction**

After incubation, the contents of each cup were filtered through whatman filter paper No.1 under vacuum. The filtrate was extracted with 25ml of chloroform in a separating funnel with gentle shaking for 1min then let until two layers separated. The lower layer (organic) was taken and evaporated to dryness in oven at 40-50°C. The residue was re-dissolved in 1ml of chloroform and the containers were stored at -20°C until use.

**TLC Test**

TLC was carried out on a silica gel 60 plate 20 × 20cm (Merck, Germany) and with modification using toluene: chloroform: acetone (15:75:10 v/v) as mobile phases [22]. AFs mix standard (20µl) (Sigma, USA) and 50µl of test samples were spotted on TLC plates and was run for 60min at room temperature. Plates were air-dried and checked over a UV Transilluminator (360nm). The AF spots were marked and the retention factor (Rf) value was calculated for each spot in comparison with the standards [23].

To make sure that there might be other types of AFs like AFG1 and AFG2 and did not appear on TLC plate. We selected best aflatoxigenic isolates that produce AFB1 and AFB2 and they were analyzed by HPLC technique using the same conditions as mentioned in Table 2.

**Aflatoxins analysis in Dried Fruit Samples**

A modified method of AFs extraction and purification was conducted according to the manual instructions [24]. Total of 10g of dried fruit sample were homogenized in a blender for 3min with 100ml of methanol: water (80:20 v/v) plus 1g NaCl was added in order to facilitate separation then it was filtrated under vacuum through Whatman filter paper NO.1. The filtrate was transferred to a separating funnel then same volume of chloroform was added and mixed gently. The organic layer was evaporated at 40-50°C in oven. The resultant dried crude extract was dissolved in 1ml chloroform and the containers were stored at -20°C for further analysis.

**Aflatoxins Determination**

**TLC Test**

TLC tests for the fruit AFs extracts were performed as mentioned above.

**Spectrophotometer**

For total AFs quantitative estimation, the dried crude extract was dried then dissolved in 1ml benzene: acetonitrile (98:2 v/v), vortexed then centrifuged at 5000xg for 5min. The optical density (OD) of AFs present in the supernatant was determined at 360nm using the information in Table 1 and calculated according to the following formula [25]. Purified spots were stored in dark at -20°C until use.

\[
\text{AFs mg/l} = \frac{D \times M}{E \times L} \times 1000
\]

D: absorbance, M: molecular weight, E: molar extinction coefficient of AFs, and L: path length (1cm).

### Table 1: Aflatoxins Data in benzene : acetonitrile (98:2)

<table>
<thead>
<tr>
<th>Aflatoxin</th>
<th>Formula</th>
<th>Mol. Wt. g/mol</th>
<th>Molar Absorptivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>AFB1</td>
<td>C₁₂H₁₇O₅</td>
<td>312</td>
<td>19,800</td>
</tr>
<tr>
<td>AFB2</td>
<td>C₁₀H₉O₅</td>
<td>314</td>
<td>20,900</td>
</tr>
<tr>
<td>AFG1</td>
<td>C₁₀H₈O₇</td>
<td>328</td>
<td>17,100</td>
</tr>
<tr>
<td>AFG2</td>
<td>C₁₀H₈O₇</td>
<td>330</td>
<td>18,200</td>
</tr>
</tbody>
</table>

**HPLC Analysis**

About 5g of Granular Activated Charcoal (GAC) were added with 10 ml of HPLC grade methanol : water (1:9 v/v). Sample was shacked at 300 rpm for 15min. Thereafter, the liquid phase was discarded by syringe and 5ml of absolute ethanol was added to the (GAC) and sonicated for 10min at room temperature. Thereafter, solvent was transferred by syringe to a new cup and evaporated at 40°C in vacuum drying oven (Guomiing, Korea). The resultant dried extract was dissolved in 1ml of HPLC grade methanol and centrifuged at 10000xg for 1min then Millipore filtered. About 20µl were
injected to the HPLC instrument (Shimadzu, Japan) [26]. The HPLC conditions with some modification are summarized in Table 2.

<table>
<thead>
<tr>
<th>Table 2: HPLC conditions [27]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Column</td>
</tr>
<tr>
<td>Mobile Phase</td>
</tr>
<tr>
<td>Flow Rate</td>
</tr>
<tr>
<td>Temp.</td>
</tr>
<tr>
<td>Detection</td>
</tr>
<tr>
<td>Injection</td>
</tr>
</tbody>
</table>

The samples were analyzed with AFs standers mix using diode-array detection (DAD) at 365nm. AFs in samples were checked depending on retention time (RT) and spectrum of each standard AF that was compared with those of each sample and AFs concentration was calculated.

**Statistical Analysis**

Statistical analysis of the data was done by using IBM SPSS software version 20, one-way ANOVA (Duncan) analysis. All p values <0.05 were considered as statically significant.

**Results and Discussion**

**Isolation of Molds from Dried Fruit Samples**

Total of 112 isolates were obtained from dried fruit samples belonging to different fungal genera where the number of A. niger 95 isolates (79%), A. flavus 15 isolates (12%) and A. terreus 2 isolates (2%). The other fungal species were 8 isolates (7%) (Figure 1). A. niger and A. flavus showed high appearance rate in dried fruit samples A. niger reached 100% in S12 and S14 while the lowest rate reached 57.8% in S11. A. flavus recorded highest rate of appearance in S11 reached 31.5% and the lowest rate of appearance reached 8.4% in S15. As a general result, A. niger appeared in high incidence in comparison with A. flavus in all fruit samples under study (Figure 2) that disagree with what was reported before [28].

![Figure 1: Ratio of as per gill us sp. incidence in dried fruit samples](image1)

![Figure 2: Percentage of A. flavus and A. niger isolates from dried fruit samples](image2)
The high incidence of *Aspergillus* genus in most food and feedstuff, reflects its ability to secrete various enzymes and some species can tolerate poor environmental conditions and grow in low moisture content in addition to high relative density of spores [29]. The mold isolates were identified morphologically as in Figure 3.

It is clearly visible that S11 Fig sample (Figure 2) “imported from Iran” contain variability of mold species (*A. niger, A. flavus* and *A. terreus*) in comparison with S12 Iraqi Fig sample that showed 100% *A. niger* incidence. This result is highly valuable in fruit origin determination, quality assessment and possible new fungal infection outbreak especially that S11 sample showed higher AFs content that must be taken in consideration (Figure 7). Table 3 shows that, despite significant difference in pH and aw within range 3.1-4.5 and 0-0.13, respectively, *A. niger* and *A. flavus* can grow successfully in all samples. There was no significant difference in *A. flavus* and *A. niger* count between samples. However, S10 sample showed significant difference with all samples (but S13) in *A. niger* count. There was no significant difference between samples S10 and S14 in pH and aw but showed significant difference *A. niger* incidence. While there was significant differences between S10 and S15 in pH, aw, mold and total *Aspergillus* isolates.

<table>
<thead>
<tr>
<th>Code</th>
<th>Plum Description</th>
<th>S10 pH</th>
<th>S11 pH</th>
<th>S12 pH</th>
<th>S13 pH</th>
<th>S14 pH</th>
<th>S15 pH</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>3.2±0.2</td>
<td>4±0.1</td>
<td>4±0.2</td>
<td>4.3±0.1</td>
<td>3.1±0.1</td>
<td>4.5±0.2</td>
</tr>
<tr>
<td></td>
<td>aw</td>
<td>0.08±0.01</td>
<td>0.1±0.01</td>
<td>0.13±0.03</td>
<td>0.03±0.01</td>
<td>0.06±0.002</td>
<td>0.0±0.002</td>
</tr>
<tr>
<td>Molds</td>
<td></td>
<td>11.3±2.08</td>
<td>7±1.7</td>
<td>4±0</td>
<td>8±1.7</td>
<td>5.3±1.5</td>
<td>4.3±0.57</td>
</tr>
<tr>
<td></td>
<td><em>A. niger</em></td>
<td>8.6±1.52</td>
<td>3.6±0.57</td>
<td>4±0</td>
<td>6±3.46</td>
<td>5.3±1.5</td>
<td>4.3±0.57</td>
</tr>
<tr>
<td></td>
<td><em>A. flavus</em></td>
<td>2±1.73</td>
<td>2±2</td>
<td>0±0</td>
<td>0±0</td>
<td>0±0</td>
<td>0.3±0.57</td>
</tr>
<tr>
<td>Total <em>Aspergillus</em></td>
<td>10.66±2.08</td>
<td>6.33±1.52</td>
<td>4±0</td>
<td>6.66±2.88</td>
<td>5.33±1.52</td>
<td>4±0</td>
<td>4.3±0.57</td>
</tr>
</tbody>
</table>

High sugar concentration and low water activity in dried fruits assist the development of *Aspergillus* species, especially *A. niger, A. carbonar* and *A. ochraceus* since they are xerophilic and *A. niger* is the most predominant mold in most dried fruit samples [28, 30-32]. *A. niger* was the most detected fungus followed by *A. flavus* in dried Fig, Apricot and plum samples [4, 32-35].
The $aw$ of dried fruit samples, facilitated the growth of xerophilic fungi and was in a range of 0 to 0.13 in dried fruit samples under study. This range disagree with [28, 32], but S11. This might be because of hot and dry conditions and or long storage time of the collected samples.

**Detection of Toxigenic Aspergillus sp. by TLC**

Total aflatoxicogenic *Aspergillus* sp. isolates from dried fruit samples were 61 isolates at rate of 54.4 % (85.24% *A. niger* and 14.75% *A. flavus*), AFB1 and AFB2 were the only AFs detected (Table 4).

<table>
<thead>
<tr>
<th>Code</th>
<th>Toxigenic sp.</th>
<th>No.</th>
<th>%</th>
<th>Aflatoxins</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>B1</td>
</tr>
<tr>
<td>S10</td>
<td><em>A. flavus</em></td>
<td>6</td>
<td>100</td>
<td>+</td>
</tr>
<tr>
<td>S11</td>
<td><em>A. niger</em></td>
<td>17</td>
<td>65</td>
<td>+</td>
</tr>
<tr>
<td>S13</td>
<td><em>A. flavus</em></td>
<td>2</td>
<td>100</td>
<td>+</td>
</tr>
<tr>
<td>S14</td>
<td><em>A. niger</em></td>
<td>16</td>
<td>100</td>
<td>+</td>
</tr>
<tr>
<td>S15</td>
<td><em>A. flavus</em></td>
<td>5</td>
<td>41</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td><em>A. flavus</em></td>
<td>1</td>
<td>100</td>
<td>+</td>
</tr>
<tr>
<td>Total Aspergillus AFs Producer</td>
<td>61</td>
<td>54.4%</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>A. niger</em></td>
<td>52</td>
<td>85.24%</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td><em>A. flavus</em></td>
<td>9</td>
<td>14.75%</td>
<td>+</td>
</tr>
</tbody>
</table>

The results in Table 4 showed that 100% of *A. niger* isolates from S11 and S14 samples and *A. flavus* from all samples, produce AFB1 and AFB2. Figure 4 showed that aflatoxicogenic isolates, *A. niger* and *A. flavus* found producing AFB1 and AFB2 only and all other dried fruit samples showed no AFB1 or AFB2 on TLC plate. The $Rf$ value for the AFB1, AFB2, AFG1 and AFG2 standards on TLC plate were 0.29, 0.23, 0.20 and 0.16 respectively.

False negative results were obtained by TLC since it is not as sensitive as HPLC for low concentration of AFs (low detection limit) less than 5ppb [37] (Figure 4). All selected isolates produced AFB1 and AFB2. Furthermore, B4N isolate produced AFG1 and AFG2 while DS1F isolate produced AFG1 (Table 4; Figure 5). This is the first report through this study that AFG is produced by both *A. niger* and *A. flavus* isolated from dried fruit.

<table>
<thead>
<tr>
<th>Molds samples</th>
<th>A flat oxin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AFB1</td>
</tr>
<tr>
<td>DS1F</td>
<td>+</td>
</tr>
<tr>
<td>D2F</td>
<td>+</td>
</tr>
<tr>
<td>D2F4</td>
<td>+</td>
</tr>
<tr>
<td>B2F</td>
<td>+</td>
</tr>
<tr>
<td>C1F</td>
<td>+</td>
</tr>
<tr>
<td>C2F</td>
<td>+</td>
</tr>
<tr>
<td>F14F</td>
<td>+</td>
</tr>
<tr>
<td>B4N</td>
<td>+</td>
</tr>
</tbody>
</table>
Figure 5: Detection of AFs production in mold using HPLC method with AFs stander (STD)

Aflatoxins Analysis in Dried Fruit Samples

Results have revealed that all samples of dried fruits were found positive with AFs. Most of the samples were above the suggested limit for total AFs 4µg/kg set by EU regulations but S12 [38]. RT for AFG2, AFG1 AFB2 and AFB1 standards were 6.8, 7.8, 9.2 and 10.6 respectively as shown in the Figures below.

Significant difference in AFs production was detected between S10 and S15. While between S10 and S12 appeared in AFB2 production. High concentration of AFB1 was detected in S15, S13, S11, S14 and S3 samples (132.3, 7.33, 2.87 and 1.59µg/kg respectively). While, AFB2 was found only in S10 and S15 (12.86 and 9.66µg/kg, respectively), AFG1 was found in S11, S14, S10 and S12 (3.5, 3, 2.69 and 1.93µg/kg, respectively), and AFG2 were found only in S13, S12 and S15 samples at 8.35, 2 and 1.4µg/kg, respectively (Table 5).

Table 5: Aflatoxins concentration (µg/kg) in Dried fruit samples by HPLC

<table>
<thead>
<tr>
<th>Code</th>
<th>S10</th>
<th>S11</th>
<th>S12</th>
<th>S13</th>
<th>S14</th>
<th>S15</th>
</tr>
</thead>
<tbody>
<tr>
<td>AFB1</td>
<td>0±0</td>
<td>2.87±0.84</td>
<td>0±0</td>
<td>7.33±0.20</td>
<td>1.59±0.53</td>
<td>132.3±0.60</td>
</tr>
<tr>
<td>AFB2</td>
<td>12.86±0.90</td>
<td>0±0</td>
<td>0±0</td>
<td>0±0</td>
<td>0±0</td>
<td>9.66±0.90</td>
</tr>
<tr>
<td>AFG1</td>
<td>2.69±0.75</td>
<td>3.5±0.50</td>
<td>1.93±1.04</td>
<td>0±0</td>
<td>3±0.55</td>
<td>0±0</td>
</tr>
<tr>
<td>AFG2</td>
<td>0±0</td>
<td>0±0</td>
<td>1.40±0.20</td>
<td>8.35±1.75</td>
<td>0±0</td>
<td>2±1.73</td>
</tr>
</tbody>
</table>

In comparison with a previous study [33] that mentioned Plum and Apricot dried fruit AFs contamination, our results showed variable results as AFB1 contamination was detected in S13 and S14 only whereas S10 and S14 samples found contaminated with AFG1 in higher concentration. Both S10 and S14 were not contaminated with AFG2. S10 found contaminated with AFB2 while S13 found contaminated with AFG2.

Figure 7: Detection of AFs in dried fruits using HPLC method with AFs stander (STD)
Dried Fig and apricot samples had shown no contamination with AFB2. AFG2 was not detected in Fig samples (S11) that agree with [4], but these samples are contaminated with AFB1 at lower concentrations than the study mentioned as shown in Table (4). However, it was reported that no AFs contamination was detected in the Fig samples [39]. This is the first report through this study that the dried Fig and apricot samples were found contaminated with AFG1 and AFG2. S11 was found contaminated with AFB1 at 2.87µg/kg that is lower than what was previously reported [30].

S10, S11 and S12 samples found contaminated with AFG1 at lower concentration as compared with previous study [40]. Apricot samples contaminated with AFs at concentration range 4.59 to15.5µg/kg which is lower than what was reported by [32, 41], but higher range as compared with [12, 42]. Dried Plum sample was found contaminated with total AFs at 15.55µg/kg, which is higher than what was previously reported [24, 32]. Despite the lack of significant differences between S10 and S12 samples in pH and aw, they were AFB1 negative. The result shows that dried fruit samples facilitate the production of AFG1 and AFG2 at pH < 6. This disagrees with a previous study [43]. The results of the present study show that the presence or absence of AFB1 and/or AFB2 is not related with aw and pH. It is most probably related with the dried fruits composition.

**Conclusion**

The mold isolates showed different AFs production behavior that they are able to produce AFB1 and AFB2 at a pH range (3.1 to 4.5) and optimum temperature at range (28-32°C) that disagree with what was reported previously [44]. This indicates that our isolates prefer acidic conditions, ambient temperature and low aw for best AFs production and this needs much concern especially with dried fruit storage and treatment conditions as reported before [5]. From our knowledge, this is the first study reporting that Jujube sample (S15) appeared contaminated with AFB1, AFB2 and AFG2 at high concentration.

**References**


