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Identification of Toxigenic Fungi Recovered from Dried Fruits

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***Abstract*: Dried fruits contain protein, carbohydrate, dietary fiber, vitamins, minerals and antioxidants. Antioxidants work to prevent cancer, stroke, heart disease, cataracts, and other diseases associated with ageing. Dried fruits can be contaminated with several kinds of fungi that produce mycotoxins such as aflatoxins, ochratoxin A, patulin and zearalenone.**

Objectives: The study aimed to determine the species of toxicogenic fungi most common on dried fruits and in living premises and to evaluate their abilities to produce secondary metabolites dangerous for human health, to evaluate mycobiota capabilities to produce mycotoxins in their cultural media, to set up a DNA-based method for detecting aflatoxigenic fungi *Aspergillus* species isolated from dried fruits using species-specific primers to increase the awareness of mycotoxins in foods by developing fast and rapid mycotoxin diagnosis kits that are easy and economical to use and enhance the awareness of mycotoxins.

Methods: 15 types of dried fruits were collected from many markets in different areas in the Kingdom of Saudi Arabia which produced in different countries. All two groups were distributed, first group were surface disinfected by dipping with sodium chloride for 3 minute and washed several times with sterilized water, after that we put sterilized fruits on Petri- dish containing Potato dextrose agar (PDA). Second group of dried fruits were put on PDA without surface sterilization. The PDA and Cazpek's-Dox agar media were used for the pathogen inoculation. For identification of fungi and their mycotoxins was used different types of methods such as single spore isolation technique, UV light screening test for mycotoxins production, ELISA, HPLC as well as PCR.

Results: Raisins (grape) gave the highest total number of fungi isolates while the lowest number of fungi isolates was given by fig and the mango and the highest number of fungi isolates was produced from the non-sterilized dry fruits, while raisins yielded the highest number of fungi, the dried Mango yielded the lowest number. Also results found that *Aspergillus niger* showed the highest isolation frequency (50.8%) and *Aspergillus flavus* isolation frequency was (19.5%).

Determination of the mycotoxins produced from 26 isolates of *Aspergillus* species which isolated from different dried fruits by ELISA, found that while *Aspergillus flavus* from coconut yield all the 6 toxins, the isolate of *Aspergillus niger* which isolated from date did not produce any kind of toxins.

The PCR was used for the detection of mycotoxigenic species. The test was performed with an optimized protocol for dried fruits which includes a set of species- specific PCR assays. Specific PCR assays were developed to detect *A. flavus* or *A. niger*. A specific detection of *A. flavus and A. niger* using DNA obtained.

Conclusion: High level of genetic variability among non-aflatoxigenic *A. flavus* isolates that require greater attention in order to design a molecular experiment to distinguish true aflatoxigenic from non-aflatoxigenic *A. flavus* strains.

***Keywords:* Fungi, Dried Fruits, mycotoxins, ELISA, HPLC, PCR.**

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I. INTRODUCTION

Dried fruits have become an increasingly attractive snack food because of containing essential amino acids, vitamins, minerals and rich dietary fibers which are beneficial for keeping health [1]. Dried fruits contain carbohydrates such as fiber and monocarbohydrates and antioxidants as flavonoids, phenolic acids, carotenoids and vitamins in concentrated form compared to fresh fruits [2]. Due to the enormous production and consumption, dried fruits are also considered an important source of various types of toxigenic fungi subject to pre- and post-harvesting, drying, handling, storage, and transport conditions [3].

Fungal infection produces various toxic metabolites called mycotoxins, Table 1. These mycotoxins have acute toxic and carcinogenic effects on human and animal health [3].

Table 1. Origin of principal mycotoxins occurring in common feeds and forages

Mycotoxins	Fungal species
Aflatoxins	*Aspergillus flavus; A. parasiticus*
Cyclopiazonic acid	*A. flavus*
Ochratoxin A	*A. ochraceus; Penicillium viridicatum; P. cyclopium*
Citrinin	*P. citrinum; P. expansum*
Patulin	*P. expansum*
Citreoviridin	*P. citreo-viride*
Deoxynivalenol	*Fusarium culmorum; F. graminearum*
T-2 toxin	*F. sporotrichioides; F. poae*
Diacetoxyscirpenol	*F. sporotrichioides; F. graminearum, F. poae*
Zearalenone	*F. culmorum; F. graminearum; F. sporotrichioides*
Fumonisins; moniliformin; fusaric acid	*F. moniliforme*
Tenuazonic acid; alternariol; alternariol methyl ether; altenuene	*Alternaria alternate*
Ergopeptine alkaloids	*Neotyphodium coenophialum*
Lolitrem alkaloids	*N. lolii*
Ergot alkaloids	*Claviceps purpurea*
Phomopsins	*Phomopsis leptostromiformis*
Sporidesmin A	*Pithomyces chartarum*

Aflatoxins (AFs) are the mycotoxins which are produced by certain strains of *Aspergillus flavus*, *Aspergillus paraciticus* and *Aspergillus nomius* [4]. AFs are usually found in cereals, milk, tree nuts and oilseeds. There are 18 different types of aflatoxins, such as B1, B2, G1, G2, M1, M2, P, Q, etc [5].

Ochratoxin is a nephrotoxic mycotoxin which may contaminate various foods and feed products worldwide. *Aspergillus niger* is one of the species responsible for ochratoxin contamination in grapes and derived products. This species has recently been split into *Aspergillus niger* and *Aspergillus welwitschiae* [6].

Fusarium mycotoxins are the most economically important fungal toxins. Fumonisins, zearalenone and trichothecenes are the major representatives of *Fusarium* mycotoxins. The *Fusarium* mycotoxins contaminate cereal grains, animal feeds and human food products, and cause huge economic losses and pose a threat to animal and human health globally [7].

The objectives of this study were:

1- To determine the species of toxicogenic fungi most common on dried fruits and in living premises and to evaluate their abilities to produce secondary metabolites dangerous for human health.

2- To evaluate mycobiota capabilities to produce mycotoxins in their cultural media.

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3- To set up a DNA-based method for detecting aflatoxigenic fungi *Aspergillus* species isolated from dried fruits using species-specific primers.

4- To increase the awareness of mycotoxins in foods by developing fast and rapid mycotoxin diagnosis kits that are easy and economical to use and enhance the awareness of mycotoxins.

II. MATERIALS AND METHODS

A. Media

Potato dextrose agar (PDA): 39 gm of PDA powder was suspended and 0.02% rose bengal in 1000 ml of distilled water. Autoclaving was sterilized at 15 lbs pressure (121°C) for 25 minutes. Under sterilized laminar flow hood, he media was cooled down then poured into sterilized Petri dishes. The plates solidify was allowed.

Cazpek's-Dox agar media: 49 gm of media was suspended in 1000 ml distilled water. Autoclaving was sterilized at 15 lbs pressure (121°C) for 15 minutes. Mixing well and casting into sterile Petri plates.

SKMY media: Prepared by the addition of 39 grams of PDA powder and 5 ml sodium chloride in 1000 distilled water. Autoclaving was sterilized at 15 lbs pressure (121°C) for 25 minutes.

B. Dried Fruit

The dried fruits samples were collected from many markets in different areas in the Kingdom of Saudi Arabia which produce in many different countries (Table 2). The samples were collected as follow:

Table 2. Names of the collected dried fruits and their countries

Dried fruit	Country
Raisins	Saudi Arabia
Lemon	Sri Lanka
Coconut	India
Date	Saudi Arabia
Fig	Turkey
Mango	Saudi Arabia
Apricot	Saudi Arabia
Papaya	South America
Banana	Malaysia
Prune (plums)	Saudi Arabia
Ananas (Pineapple)	Philippines
Strawberry	Philippines
Cherry	Turkey
Kiwifruit	Saudi Arabia
Pomegranate	Saudi Arabia

C. Isolation, characterization and identification of mycoflora isolated from dried fruits

Isolation of mycoflora

All the dry fruits used in this experiment were apparently free from physical damage and diseases. Dried fruits were divided into two groups; First group were surface disinfected by dipping with sodium hypochloride (1%) for 3 minute and washed several times with sterilized water, and air, after that sterilized fruits were put on Petri- dish content PDA [8]. Second group of dried fruits were put on PDA without surface sterilization.

Pathogen inoculums

PDA and Cazpek's-Dox agar media were used for the pathogen inoculation. Rose Bengal and Chloramphenicol were added to these medias as bacteriostatic antimicrobial to prevent the growth of any bacteria or other microorganisms other than fungi.

Fungi isolated from the dried fruits by taking 50 gm of every sample and cultured in 3 plates of PDA and Cazpek's-Dox agar media. These plates were incubated at 25 ºC for 7-15 days. After that, the fungal growth and the colony appearance were examined for the isolation and identification of the fungi.

After that and for the purification of the fungi, sub-cultured every single fungal colony in a separate PDA media and incubated it at 25 ºC for 7-15 days. All the isolated fungi were preserved in tubes containing PDA (slant) stored at 4 ºC, to be used during the study.

Fungi Isolation and Identification

Isolated fungi were done both macroscopically and microscopically. Fungal colonies were selected for identification, according to the methods proposed for the genus. The distinct colonies were picked; sub cultured for purification and characterized using standard techniques. The single spore isolation technique was used for the purification of the fungi. The spores are transferred to one portion of the agar plate which is then streaked across with an inoculation loop to another portion of the plate in order to separate individual spores and make the single colonies available for isolation. After this purification technique, the fungi were sub-cultured again on PDA and incubated at 25 ºC for 7-10 days. Then, it was identified using the morphological identification under the light microscope. Slide cultured colonies were stained with lactophenol blue. Taxonomic identification of the fungi was made based on macroscopic and microscopic features in accordance with appropriate keys [9, 10] identification of all the fungi were done. The incidence of occurrence of each fungus was calculated using a formula [11].

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:

% of occurrence = X/N × 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 using UV Light

The Ultra Violet (UV) 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.

D. Quantitative analysis of fungal mycotoxins

Enzyme-linked immunosorbent assay (ELISA) and High-Performance Liquid Chromatography (HPLC) were used for the quantitative analysis of the mycotoxins.

Detection of *Alternaria* toxins production

Sample preparation: Flasks were made up containing 12.5 g of autoclaved polished rice at 40% moisture. Flasks were inoculated with agar plugs of one-week-old cultures of *Alternaria* spp. isolate. The flasks were incubated in the dark at 25 ºC for 21 days [12].

Extraction of Alternaria toxins: The method for the detection of *Alternaria* toxins in rice was described by Li et al. [12]. The culture material was homogenized with 30 ml of methanol and filtered through a Whatman filter paper (no. 1). The filtrate was clarified with 60 ml of 20% ammonium sulphate. Culture filtrate was extracted three times with 10 ml of chloroform. The organic phases were combined, evaporated to dryness, and dissolved in 4 ml of methanol for AE, and AOH analysis by HPLC.

HPLC detection

HPLC system consisted of a Shimadzu liquid chromatography (Shimadzu, Kyoto, Japan) equipped with a Shimadzu SPD-M10Avp UV photodiode array detector. The analytical column was ODS 4.6x250 mm 5 u. C18.

Standards of AE and AOH were purchased from SIGMA Chemical Company (St. Louis, MO, USA). The mobile phase was methanol/water (80:20) containing 300 mg ZnSO4-H2O/l, for AE, AOH and AME. A flow rate of 0.7 ml/min was used. The wavelength for recording chromatograms was 258 nm for AE and AOH According to Scott and Kanhere [13].

***Fusarium* mycotoxins analysis**

Fusarium mycotoxins (Fumonisin, HT-2, Zearalenone, T-2, Neosolaniol and DON) content was determined using the VICAM method [14]. The method was similar with all former toxins except the dilution buffer, developer and immunoaffinity column.

Each isolate was grown in elmnyer flask 100 ml on SMKY media. The incubation period was 7 days at 25 °C. After blending on high speed for 1 min. with 5 g of sodium chloride. 20 ml of culture filtrate was added to 80 ml of methanol (HPLC grade) and filtered through a fluted filter paper. The extract (10 ml) was diluted with 40 ml of phosphate buffered saline (PBS)/0.1% Tween-20 wash buffer and filtered through a 1.0-Am microfiber filter. The diluted extract was passed through the column, which was washed with 10 ml of PBS/0.1% Tween-20 wash buffer followed by 10 ml of PBS. Fumonisins were eluted from the column with 1 ml HPLC grade methanol. A mixture of developer A and developer B (1 ml) was added to the elute collected in a cuvette that was placed in a fluorometer (VICAM Fluorometer Series 4, Watertown, USA) for fumonisin measurement.

Aflatoxin quantitative analysis in samples by indirect competitive ELISA

Sample preparations: All tested isolates were grown on SKMY Media for 10 days at 25 °C. After incubation period all flasks content was blended on high speed for 2-3 min with 5 gram of sodium chloride and then filtrate on glass filter paper. 100 ml of fungal filtrate were centrifuged at 3500 g for 10 min at 4 °C. The upper layer was removed, and samples were further diluted 20 times (v/v) with deionized water. The suspension was filtered (Millipore, 0.45 µm) and filtrate was centrifuged at 2700 g for 15 min at 15 °C, and the upper phase was removed and an aqueous-methanol layer (100 µl) was added to 0.01 M PBS (900 µl, dilution 1:10). AF content was analytically determined on 100 µl of this solution.

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 microliters of each sample were 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; according to Deshpande [15] and Thirumala-Devi et al. [16].

E. DNA extraction and PCR amplification

Genomic DNA of the strains was obtained using the genomic DNA Extraction Kit (Genomix, Talent, Trieste, Italy) following the manufacturer's instructions. All genomic DNAs used in this work were tested for suitability for PCR amplification using primers ITS1 and ITS4, which amplify the ITS region in *Aspergillus*. The PCR reaction was performed in an Eppendorf Mastercycler Gradient (Eppendorf, Hamburg, Germany) using between 10 pg and 10 ng of genomic DNA. The amplification program used was described by Henry et al. [17]. The amplification products were isolated by the High Pure PCR Product Purification Kit (Roche, Germany) and were sequenced using the ABI PRISM DNA Sequencer (Applied Biosystems, Foster City, USA) according to the manufacturer's instructions in the Genomic Unit of the University Complutense of Madrid (Spain). All the strains were sequenced in both directions. Sequences were analysed and aligned by Clustal method using the program DNAstar (Lasergene, Wisconsin, USA). PCR assays were carried out using two sets of primers: AFIJ-F/AFIJ-R (5CTTCCTTAGGGGTGGCACAGC3 and 5GTTGCTTTTCAGCGTCGGCC3, respectively) for *A. flavus* and ANIG1/ANIG2 (5GCATCTCTGCCCCTCGG3 and 5GGTTGGAGTTGTCGGCAG3, respectively) for *A. niger*. PCR reactions were performed in an Eppendorf Mastercycler Gradient (Eppendorf). The PCR amplification protocol used for *A. flavus* was as follows: 1 cycle of 4 min 30 s at 95 °C, 30 cycles of 30 s at 95 °C (denaturalization), 30 s at 63 8C (annealing), 1 min at 72 °C (extension) and finally 1 cycle of 3

min at 72 °C. In the case of *A. niger*, the PCR program was: 1 cycle of 4 min 30 s at 95 °C, 25 cycles of 30 s at 95 °C (denaturalization), 25 s at 59 °C (annealing), 40 s at 72 °C (extension) and finally 1 cycle of 5 min at 72 °C .In both case, amplification reactions were carried out in volumes of 25 AL containing 3 AL (10 pg–10 ng) of template DNA, 1.25 AL of each primer (20 AM), 2.5 AL of 10_ PCR buffer, 1 AL of MgCl2 (50 mM), 0.25 AL of dNTPs (100 mM) and 0.2 AL of Taq DNA polymerase (5 U/AL) supplied by the manufacturer (Ecogen, Barcelona, Spain). PCR products were detected in 2% agarose ethidium bromide gels in TAE 1_ buffer (Tris–acetate 40 mM and EDTA 1.0 mM). The DNA ladder bReal escala no. 2Q (Durviz, Valencia, Spain) was used as molecular size marker.

F. Statistical analysis

The experimental design used was randomized complete block design. Analysis of variance (ANOVA) was used to compare the mean and Duncan's multiple range test (DMRT) was used to separate the means [11].

III. RESULTS

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

Table 3 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 11 were given by fig and the mango. The data in table 3 explains also that 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 dried fruits the number of fungi isolates which isolated from the surface of sterilized dried 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 but in the non-sterilized pineapple the isolates were only 3.

Table 3. Isolation fungi from Sterilized and non- Sterilized dried fruits

Fruit	Sterilized	non- Sterilized	Total of isolates
Raisins	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
Ananas (Pineapple)	11	3	14
Strawberry	6	6	12
Cherry	6	7	13
Kiwifruit	2	11	13
Pomegranate	9	7	16

Percentage of fungal profiles isolated from dried fruits

Table 4 showed that the isolated fungi from dry fruits were *Aspergillus niger*, *Macrophomina phaseolina*, *Alternaria alternata*, *Penicillium digitatum*, *Penicilium itailicum*, *Aspergillus terreus*, *Fusarium chlamydosporum*, *Aspergillus wenti*, *Cladosporium herbarum* and *Aspergillius flavus.* Raisins (Grape) yielded the highest number of fungi, while dried Mango lowest yielded the lowest number. *Aspergillus niger* showed the highest isolation ferquency (50.8 %) and *Aspergillus 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 5 and Figure 1.

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Table 4. Percentage of fungal profiles isolated from dried fruits

Fungus / Fruit	*Alternaria alternate*	*Aspergillus niger*	*Macrophomia phaseolina*	*Penicilluium itailcum*	*Penicilluium digitatum*	*Cladosporium herbarum*	*Fusarium chlamydosporum*	*Aspergillus flavus*	*Aspergillus terreus*	*Aspergillus wentii*
Raisins	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	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 (plums)	0.0	100	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Ananas (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	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

Figure 1. Frequency of fungi isolates from dried fruits

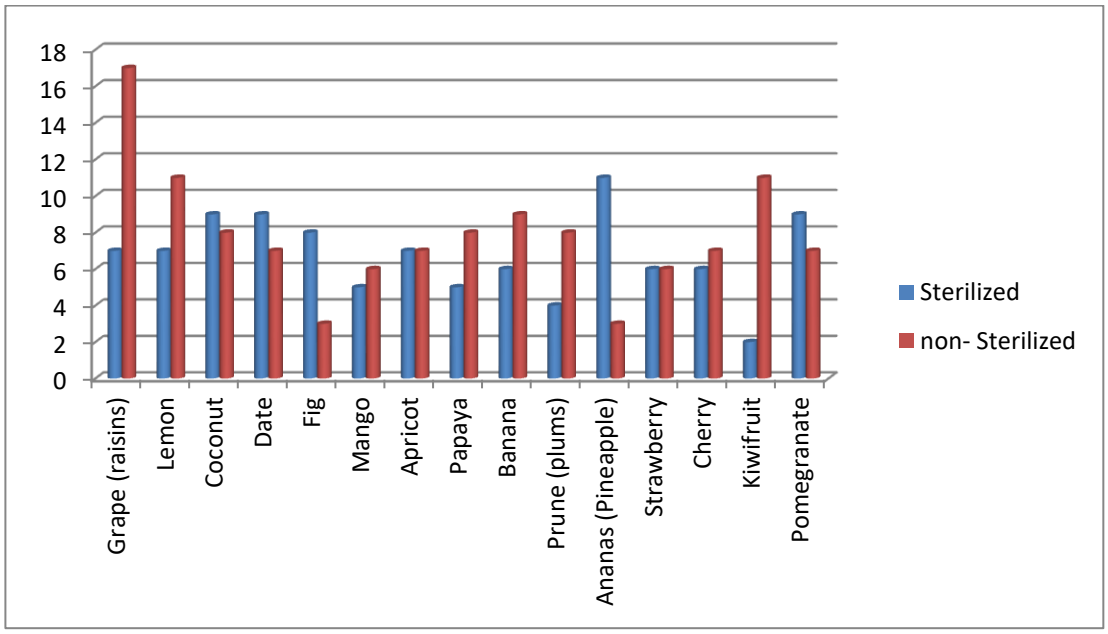

Table 5. Frequency of fungi isolated from dried fruits

Fungus	**Isolation Frequency (%)[a]**
Aspergillus niger	50.8
Alternaria alternata	2.5
Penicillium digitatum	10.4
Penicilium itailicum	2.8
Macrophomina phaseolina	0.3
Aspergillus wentii	1.8
Aspergillus terreus	2.2
Aspergillus flavus	19.5
Fusarium chlamydosporum	4.6
Cladosporium herbarum	5.6

[a] Each value is the mean of 15 replicates (fruits) percentage data were transformed into √ X+ 0.5 before carrying out ANOVA to produce approximately constant variance.

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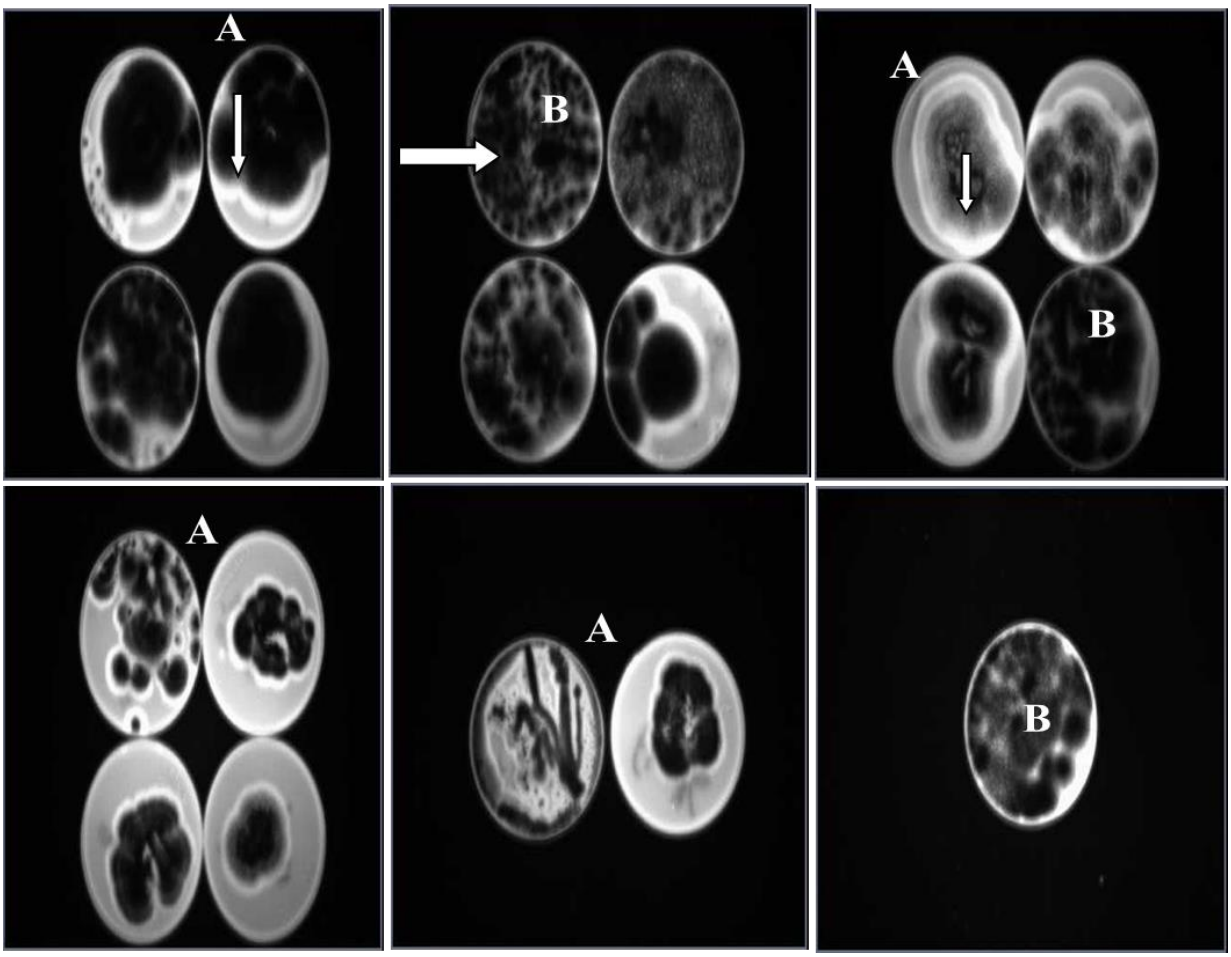
Mycotoxins producing screening test by UV Light

The UV light was used 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), Figure 2.

Figure 2. Mycotoxins producing screening test by UV Light. A. Blue green light; B. dark light



Quantitative analysis of fungal mycotoxins

The mycotoxin produced from 26 isolates *Aspergillus sp* which isolated from different dried fruits were determinated by using indirect competitive ELISA. Six important and danger toxins produced by *Aspergillus spp* for each isolate were tested, these toxins were identified as AFB1, AFB2, AFG1, AFG2, Fumagillin and Maltoryzine.

Table 6 showed that the isolate of *Aspergillus niger* which isolated from date did not produce any kind of toxins on opposite of *Aspergillus flavus* from coconut which yield all the 6 toxins by little amount of toxins compare 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.

Aspergillus flavus which isolated from raisins gave a highly concentration of mycotoxin B1, B2, G1, G2 and Maltoryzine receptively 7.75, 6.2, 9.61, 4.03 and 4.4 and so two isolates of *Aspergillus flavus* which isolated from date and dry lemon gave highly concentration of mycotoxin B1, B2, G1, G2 and Maltoryzin too. The data in table 6 explain the most of isolates of table can produce two or three mycotoxins at last. *Aspergillus niger* from dry strawberry produce only one toxin.

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Table 6. Determination of mycotoxins produced by *Aspergillus sp* by indirect competitive ELISA

			Mycotoxins (µg)					
No.	**Dried Fruit**	**Isolate**	**B1**	**B2**	**G1**	**G2**	**Fumagilin**	**Maltoryzine**
1	Raisins	*Aspergillus flavus*	7.75	6.2	9.61	4.03	0.0	4.4
2	Date	*Aspergillus flavus*	6.2	3.4	2.3	2.7	0.0	2.1
3	Date	*Aspergillus flavus*	4.1	4.7	0.0	0.0	0.1	1.9
4	Lemon	*Aspergillus flavus*	6.8	11.4	5.2	7.4	0.0	5.0
5	Date	*Aspergillus flavus*	3.5	5.3	8.9	2.7	0.0	3.3
6	Strawberry	*Aspergillus flavus*	4.9	8.9	9.6	10.2	0.0	1.2
7	Plums	*Aspergillus flavus*	10.2	4.3	3.5	2.3	0.0	0.9
8	Coconut	*Aspergillus flavus*	1.9	2.2	3.1	0.6	0.4	3.8
9	Date	*Aspergillus niger*	-	-	-	-	-	-
10	Banana	*Aspergillus wentii*	0.8	0.2	0.1	0.0	11.2	2.0
11	Prune	*Aspergillus niger*	0.6	0.4	0.2	0.1	9.9	0.7
12	Date	*Aspergillus niger*	0.1	0.2	0.0	0.1	0.0	4.2
13	Fig	*Aspergillus niger*	0.2	0.1	0.4	0.0	0.0	3.3
14	Kiwifruit	*Aspergillus niger*	0.0	0.0	0.0	0.0	0.0	1.4
15	Date	*Aspergillus niger*	0.1	0.4	0.0	0.2	0.2	0.9
16	Date	*Aspergillus niger*	0.2	0.1	0.0	0.0	0.0	1.7
17	Raisins	*Aspergillus niger*	0.3	0.0	0.0	0.1	0.3	3.2
18	Mango	*Aspergillus niger*	0.2	0.0	0.2	0.2	0.0	0.4
19	Raisins	*Aspergillus niger*	0.1	0.1	0.0	0.0	0.4	2.6
20	Lemon	*Aspergillus niger*	0.1	0.0	0.1	0.2	0.1	4.1
21	Cherry	*Aspergillus niger*	0.0	0.0	0.0	0.0	0.2	3.8
22	Strawberry	*Aspergillus niger*	0.0	0.0	0.0	0.0	0.0	5.1
23	Ananas	*Aspergillus niger*	0.4	0.2	0.3	0.0	0.0	6.3
24	Coconut	*Aspergillus niger*	0.6	0.1	0.2	0.1	0.2	0.9
25	Apricot	*Aspergillus niger*	0.1	0.1	0.0	0.3	0.0	4.6
26	Ananas	*Aspergillus flavus*	0.1	0.1	0.2	0.1	0.1	3.7

Correlation among in toxins production by isolates of *Aspergillus niger*

Table 7 found 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 8 presented the correlation among in toxins production by isolates of *Aspergillus flavus*. G1 was positively correlated with each of B2 ($p<0.05$) and G2 ($p<0.05$). All the correlations were non-significant.

Table 7. Correlation among in toxins production by isolates of *Aspergillus niger*

Toxins	**Toxins**				
	B1	**B2**	**G2**	**G1**	**Fumagillin**
B1					
B2	0.429				
G2	0.037	0.174			
G1	0.572*	0.163	-0.115		
Fumagillin	0.0.568*	0.586*	0.034	0.206	
Maltoryzine	-0.296	-0.245	-0.152	-0.04	-0.062

A personal correlation coefficient was significant at $p <0.05$(*)

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Table 8. Correlation among in toxins production by isolates of *Aspergillus flavus*

Toxins	Toxins				
	B1	**B2**	**G1**	**G2**	**Fumagillin**
B1					
B2	0.493				
G1	0.314	0.594			
G2	0.361	0.850**[a]	0.696*		
Fumagillin	-0.567	-0.484	-0.385	-0.480	
Maltoryzine	-0.272	0.133	0.129	-0.037	0.021

[a] pearsonals correlation coefficient was significant of p<0.01(**) or p<0.05 (*)

Efficiency of *Aspergillus flavus* and *Aspergillus niger* in producing mycotoxins in dried fruits

Data showed that *A.flavus* was efficient than *A.niger* in producing G1, G2, B1 and B2. The differences between *A.flavus* and *A.niger* in producing fumagllin and maltoryzine were not significant, Table 9.

Table 9. Efficiency of *Aspergillus flavus* and *Aspergillus niger* in production mycotoxins in dried fruits

Fungi	Toxins					
	G1	**G2**	**B1**	**B2**	**Fumgaillin**	**Maltoryzine**
Aspergillus flavus	4.711 [a]	3.3367	5.0889	5.1667	0.0667	2.9222
Aspergillus niger	0.0933 [b]	0.0867	0.2000	0.113	0.7533	2.8800
F .value	47.207	22.053	23.515	18.627	2.339	0.814
P>F	0.00	0.00	0.00	0.00	0.140	0.377

[a] Mean of 9 replicates (isolates); [b] Mean of 15 replicates (isolates)

Determination of mycotoxins produced by *Fusarium chlamydosporum* by using HPLC

The results of isolation from dried fruits gave three isolates from species fungi *Fusarium chlamydosporum* one isolate from dry fig and tow isolates from raisins, species *Fusarium* can produce many mycotoxins auch as fumonisin, zearalenone, DON, T-2, neosolaniol and HT-2. Data in table 10 explain the isolate No 1 of *Fusarium chlamydosporum* produce 650 µg of Fumonisin but isolates No 2and 3 gave 400 and 550 µg. the three isolates of *Fusarium chlamydosporum* were no significant different in produce mycotoxin zearalenone on the other hand mycotoxin DON can produce by isolate No 3 which isolated from fig as a result of concentration 600 µg, while isolates No 1 and 2 were 100 µg and 155 µg receptively. The data showed that too there were non-significant difference in produce mycotoxin aeosolaniol, T-2 and HT-2.

Table 10. Determination of mycotoxins produced by *Fusarium chlamydosporum* by HPLC

NO.	Dried fruits	Isolates	Mycotoxin (µg)					
			Fumonisin	**Zearalenone**	**DON**	**T-2**	**Neosolaniol**	**HT-2**
1	Raisins	*Fusarium chlamydosporum*	650	75	100	4.0	2.3	0.9
2	Raisins	*Fusarium chlamydosporum*	400	80	155	11.0	1.1	2.4
3	Fig	*Fusarium chlamydosporum*	550	90	600	14.0	4.3	1.3

Determination of mycotoxins produced by *Penicillium* by using HPLC

The results of isolation from dried fruits gave three isolates from species fungi *Penicillium* one isolate identified as *Penicillium itailcum* that isolated from raisins and tow isolates *Penicillium digitatum* No 2 from fig and No 3 from pomegranate. *Penicillium* can producing many mycotoxin auch as penicillic acid, citreoviridin, citrinin and patulin. The resulted showed that citrinin can produce by *Penicillium itailcum* by concentration 6 µg other *Penicillium digitatum* No 2 and 3 were 9 µg and 4 µg. the three isolates of *Penicillium* were not significant in produce other mycotoins, Table 11.

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Table 11. Determination of mycotoxins produced by *Penicillium* by using HPLC

NO.	Dried Fruits	Isolates	Mycotoxin (µg)			
			Penicillic acid	citreoviridin	Citrinin	Patulin
1	Raisins	*Penicillium itailcum*	1.2	11	6	18
2	Fig	*Penicillium digitatum*	1.o	9	9	24
3	Pomegranate	*Penicillium digitatum*	0.9	6	4	16

Determination of mycotoxins produced by *Alternaria sp* by using HPLC

Table 12 presented the fungi *Alternaria alternata* which isolated from date and raisins can produce mycototins Altenuene and Alternariol by concentration 49, 32 for Altenuene and 25 and 19 for Alternariol.

Table 12. Determination of mycotoxins produced by *Alternaria sp* by HPLC

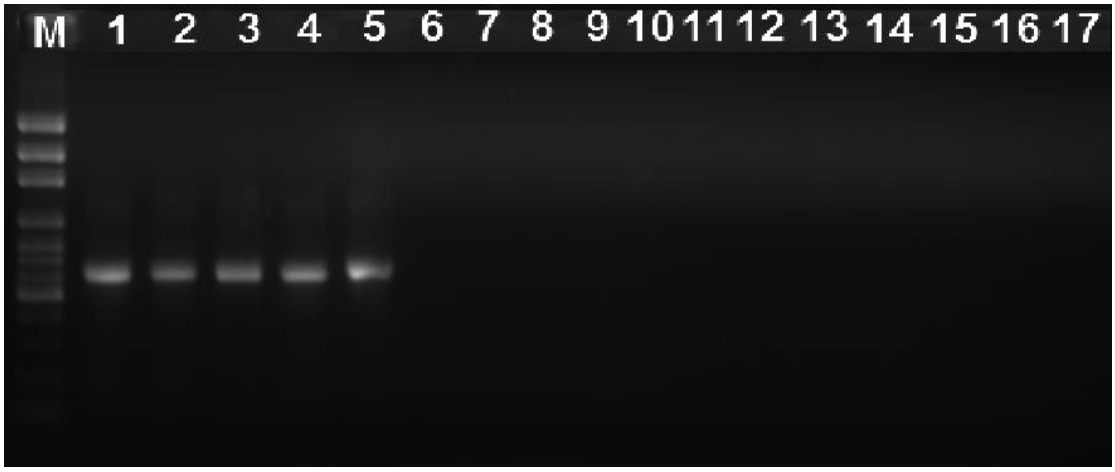
NO.	Dried Fruits	Isolates	Mycotoxin (µg)	
			Altenuene	Alternariol
1	Date	*Alternaria alternata*	49	25
2	Raisins	*Alternaria alternata*	32	19

Molecular chractarization of some toxigenic fungi (*Aspergillus sp*)

Detection of these mycotoxigenic species was performed with an optimized protocol for dried fruits which includes a set of species- specific PCR assays. Amplification products, regardless of the primer set used, were obtained from *Aspergillus* isolates DNA. Specific PCR assays were developed to detect *A. flavus* or *A. niger* using primers based on the multicopy ITS2 rDNA target sequence. The species specificity of the assays was tested in a wide range of strains of these species and others colonizing the same commodities. A single DNA fragment with expected size of 840 bp was amplified by aflJ-F and aflJ-R. *primer pair in 5 isolates of A. flavus* (all isolates was aflatoxinogenic isolates), Figure 3. A non-intensity band was seen with ANIG1 and ANIG2 and A. niger DNA when 40 PCR cycles were applied. Applying this primer pair resulted in no amplification in twelve *Aspergillus niger* isolates. used as control in this study The DNA of *A. terrus* and *A. wentii* were also subjected to PCR using ANIG primers, but no amplicons were observed.

Figure 4 presented PCR amplification using primers AFLr1 and AFLr2 while Figure 5 showed PCR-based detection method specific to *Aspergillus niger* using primers anig1 – anig 2. PCR amplification specific to *Alternaria Alternata* using primers ALTF/ALTR was exhibited in Figure 6.

Figure 3. PCR-based detection method specific to *Aspergillus flavus* using primers aflJ-F and aflJ-R



Specific detection of *A. flavus* using DNA obtained from culture and the primer pair aflJ and F-aflJ-R. M; Solis BioDyne DNA Marker 100 bp, Lanes 1–5: *A. flavus*, Lanes 6-15 *A. niger*, Lane 16 *A. terrus*, Lane 17 and Lane *A. wentii*.

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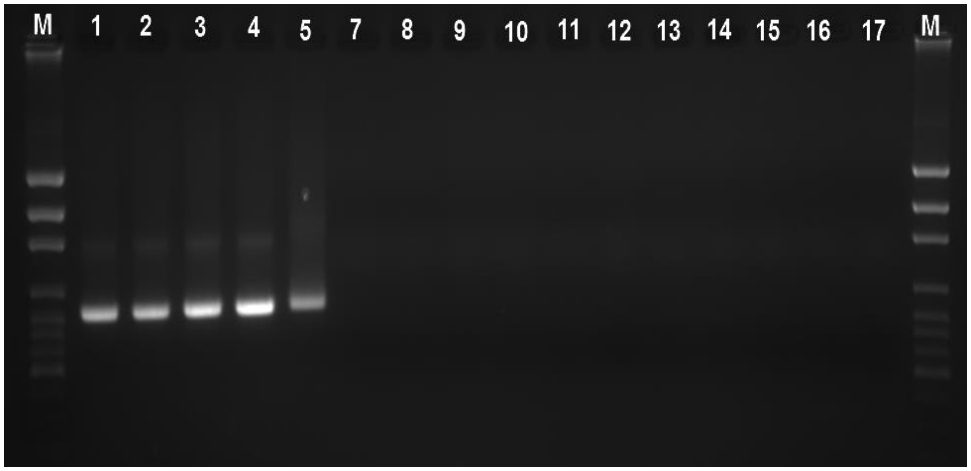
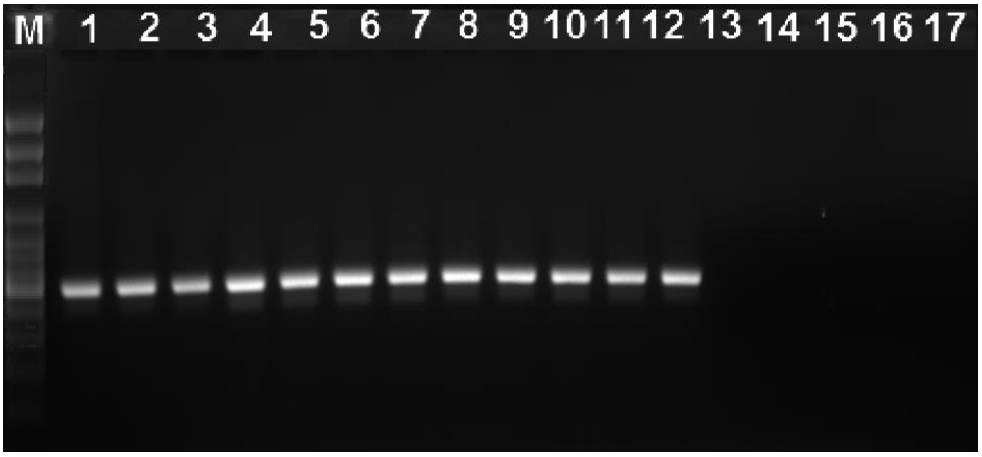
Figure 4. PCR amplification using primers AFLr1 and AFLr2



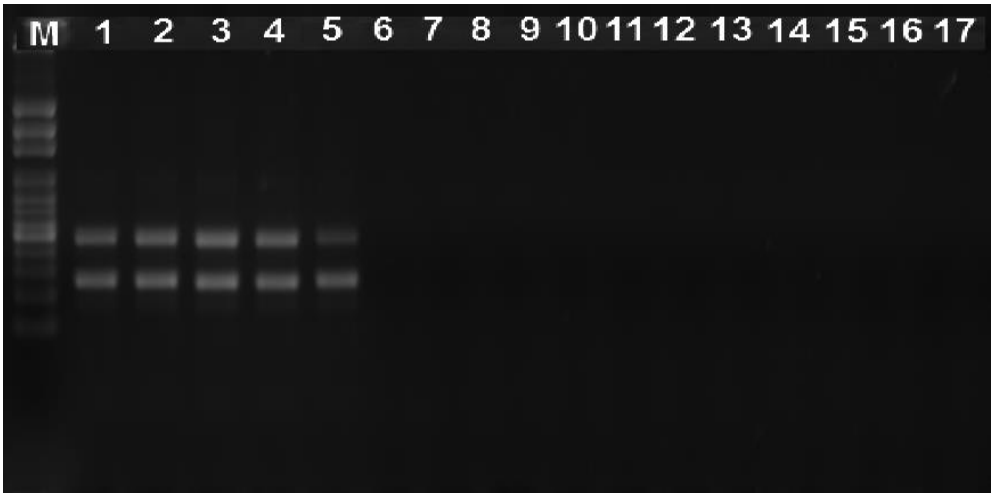
Specific detection of *A. flavus* using DNA obtained from culture and the primer pair AFLr1 and AFLr2. M; Solis BioDyne DNA Marker 100 bp, Lanes 1–5: *A. flavus*, Lanes 6-15 *A. niger*, Lane 16 *A. terrus*, Lane 17 and Lane *A. wentii*.

Figure 5. PCR-based detection method specific to *Aspergillus niger* using primers anig1 – anig 2



Specific detection of *A. niger* using DNA obtained from culture and the primer pair ANIG1 and ANIG2. M; Solis BioDyne DNA Marker 100 bp, Lanes 1–12: *A. niger*, Lanes 13-15 *A. flavus*, Lane 16 *A. terreus*, Lane 17 and Lane *A. wentii*

Figure 6. PCR-based detection method specific to *Alternaria Alternata* using primers ALTF/ALTR



PCR amplification using primers ALTF/ALTR and DNA from *Alternaria Alternata* isolates. Lanes 1-7: Lanes: M - 100 bp DNA Marker (Solis BioDyne).

IV. DISCUSSION

The present study examined 15 types of dried fruits to isolate and identification the fungi and its toxins. The results 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 11 were given by fig and the mango, in contrary to these findings, among all fruits tested by Tournas and Katsoudas, berries had the highest levels of contamination; strawberries (97% 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 [18].

The data showed also that there is different in numbers of isolated fungi from sterilized dry fruits and non-sterilized dry fruits, the highest number of fungi isolates were produced from the non-sterilized dry fruits, 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. In opposite to these findings, found that 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 such as in figs and pineapples (8-3, 11-3). Also, found no significant differences between sterilized and non-sterilized dry fruits such as Apricot, strawberry and cherry (7-7, 6-6 and 6-7, respectively).

While raisins (grape) yielded the highest number of fungi, dried Mango yielded the lowest number. In agreement to other studies [19, 20] found that *Aspergillus niger* showed the highest isolation ferquency (50.8%) and *Aspergillus flavus* isolation frequency was (19.5%).

The other studies demonstrated that the species *A. niger var. niger* and *Aspergillus niger var. awamori* were isolated in higher frequency from dried fruits. Also, there were no significant differences in isolation frequency between the other fungi which showed isolation frequencies ranged from 0.3 to 10.4 %.

Dry weights of mycelium were used in some species fungi isolated from dry fruits in liquid media. It showed that there were no significant differences in dry weights of mycelium of fungi between each other fungi which produce mycotoxins, but some isolates of fungi gave highly dry weight such as *Aspergillus wentii*, *Alternaria alternata* 2 and *Aspergillus niger* 10 were 1.7 gm for the three isolates fungi. On the other hand, *Aspergillus flavus* 5 and *Penicillium itailcum* were the lowest dry weight was 0.94 gm.

UV light for the screening of all the fungi was used to identify which isolates produce mycotoxins. Found that 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).

These findings supported by the work of Sekar et al in India, they proved the efficacy of the aflatoxin-screening processes by UV-light [21].

In the present research work, the mycotoxin (AFB1, AFB2, AFG1, AFG2, Fumagillin and Maltoryzine) produced from 26 isolates of *Aspergillus* species which isolated from different dried fruits was detramined by using ELISA. The results showed that *Aspergillus flavus* from coconut yield all the 6 toxins, while the isolate of *Aspergillus niger* which isolated from date did not produce any kind of toxins, the same findings obtained by other study [22], but Ahmed et al 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 as they explained [23].

Also found that the toxin Maltoryzine was produced by completely all isolates of *Aspergillus spp* except for one isolate. *Aspergillus flavus* which isolated from raisins gave a highly concentration of mycotoxin B1, B2, G1, G2 and maltoryzine while other study found only B1 [19] may be due to their limited methods and samples. Also, two isolates of *Aspergillus flavus* which remote from date and dry lemon gave highly concentration of mycotoxin B1, B2, G1, G2 and maltoryzin too.

Found that toxin B1 produced by *Aspergillus niger* was positively correlated with each of G1 ($p<0.05$) and fumagillin was positively correlated with each of B1 ($p<0.05$) and B2 ($p<0.05$). While all the other correlations were non-significant. The results showed that correlation in toxins produced by isolates of *Aspergillus flavus*. G1 was positively correlated with each of B2 ($p<0.05$) and G2 ($p<0.05$).

Using HPLC, determined mycotoxins produce by *Fusarium chlamydosporum* and found three isolates from these fungi, one isolate from dry fig and two isolates from raisins. Species *Fusarium* can produce many mycotoxins such as fumonisin, zearalenone, DON, T-2, neosolaniol and HT-2. Same method used with *Penicillium*, the results of isolation from dried fruits gave three isolates from species fungi *Penicillium* one isolate identified as *Penicillium itailcum* that isolated from raisins and two isolates *Penicillium digitatum* from fig and pomegranate. *Penicillium* can produce many mycotoxins such as penicillic acid, citreoviridin, citrinin and patulin. The results showed that fungi *Alternaria alternata* which isolated from date and raisins can produce mycotoxins altenuene and alternariol. This species found to be predominant compared to other fungi in study performed by Chulze et al in Argentina [24].

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.

Black-spored *aspergilli* are difficult to classify and the taxonomy of this section is still unclear [25]. Traditionally, the classification of this section was based on morphological characteristics. The difficulty of identifying phenotypic, a diagnostic phenotypic procedure based on biochemical traits on agar media along with some molecular approaches has been recently reviewed [26].

Nested PCR was used mainly to confirm the authenticity of the primary PCR. The detection limit and incubation time to detect *A. flavus* and *A. niger* in our study was less than the reported by other workers. No amplification was observed with *A. terrus* and *A. wentii* tested, which may be due to lack of complimentary regions in the primer binding regions.

In this work, the detection of toxigenic *Aspergillus* species in market dried fruits was performed using a set of species-pecific PCR assays. Additionally, genomic DNA extraction method was optimized for both matrices to yield DNA of PCR quality to be used for routine analysis of high number of samples. Most of the fungi belonging to *A. flavus* group reacted positively with the primers resulting in expected size amplicons of 796 bp for AFLR and 404 bp for ANIG.

The data showed a high level of genetic variability among non-aflatoxigenic *A. flavus* isolates that require greater attention in order to design molecular experiment to distinguish true aflatoxigenic from non-aflatoxigenic *A. flavus* strains.

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