

# SCREENING OF AFLATOXIN-PRODUCING MOULD ISOLATES BASED ON FLUORESCENCE PRODUCTION ON A SPECIFIC MEDIUM UNDER ULTRAVIOLET LIGHT

M. Razzaghi abyaneh<sup>1</sup>, A. Allameh<sup>2</sup> and M. Shams<sup>1</sup>

Departments of Mycology<sup>1</sup> and Biochemistry<sup>2</sup>, Faculty of Medical Sciences, Tarbiat Modarres University, Tehran, Iran

**Abstract** - Aflatoxin contamination of food and feedstuffs is a serious economic and health hazard. Over a period of two years, 35 samples of raw pistachio nuts collected from different parts of Iran were analysed for the presence of aflatoxigenic strains of the *Aspergillus flavus* group. Eighty-five isolates of *A. flavus* and *A. parasiticus* were identified based on specific yellow-orange pigment production in the reverse of the colonies on *Aspergillus flavus* and *parasiticus* agar (AFPA) medium and microscopic criteria. These isolates were present in 65.71% of the samples and their aflatoxin-producing ability was examined. At first, 12 out of the 15 aflatoxin-producing isolates were selected based on bright blue fluorescence colour on aflatoxin-producing ability (APA) medium. Later, all of the isolates were identified after culturing on sucrose-low salts (SLS) medium. Furthermore, aflatoxin production on APA and SLS media was confirmed when the mycelia and media were extracted and analysed for aflatoxins on TLC plates. *A. flavus* strains produced mainly aflatoxin B<sub>1</sub> with concentrations ranging from 0.21 to 1.49 µg/g of APA medium, where as *A. parasiticus* strain produced all four aflatoxins (B<sub>1</sub>, B<sub>2</sub>, G<sub>1</sub> and G<sub>2</sub>) with 3.71 µg aflatoxin B<sub>1</sub>/g of the medium. Isolates other than *A. flavus* and *A. parasiticus* grown on this specific culture medium failed to produce a characteristic fluorescence under UV light. Evidences from this study indicate that fluorescent production on APA medium is a reliable screening test for distinguishing potential aflatoxin-producing strains of *A. flavus* and *A. parasiticus* from nontoxigenic strains.

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**Key Words:** Aflatoxin, fluorescence, pistachio nut, *Aspergillus*, specific medium

## INTRODUCTION

Aflatoxins are secondary metabolites produced by certain strains of the common moulds *Aspergillus flavus*, *Aspergillus parasiticus*, *Aspergillus nomius* and *Aspergillus tamaris* (1-3). These toxins with polyketide origin (Fig. 1) have been reported to be highly toxic,

teratogenic, carcinogenic and mutagenic agents, which occur naturally in a wide variety of feed and feedstuffs (4,5). Under favorable conditions, toxigenic fungi grow on various agricultural commodities and produce mycotoxins e.g. aflatoxins (6,7). Outbreaks of aflatoxin poisoning in human (liver cancer) and animals (decreased weight gain, hemorrhaging and suppression of the immune system) are closely related to consumption of foods and feeds contaminated with aflatoxin-producing strains (8).

Different authors have reported a number of screening methods for the detection of aflatoxin-producing moulds and most of them have used various media, large cultures and long incubation periods, which can involve considerable time, work and expense (9). These methods can be mainly divided in to three groups. The first group includes standard methods which are based on toxin production on both liquid or solid substrates, followed by purification procedures and final detection by thin-layer chromatography or high performance liquid chromatography (10, 11). In the second group, a small agar plug from a pure mould culture is used directly to apply the sample onto TLC-plate (12). Although this method is simple, it has disadvantages such as scattering of fungal spores during the operation. The third group includes methods for the detection of fluorescence from aflatoxins in liquid culture or an agar plate under the ultraviolet (UV) light (13-18). Generally, classic analytical methods are expensive and need sophisticated equipments and take long period of time for setup. However, screening of the fungi directly on a culture medium is simple and relatively fast procedure.

This study was undertaken to develop a method based on the formation of bright blue fluorescence of the agar medium surrounding colonies under UV light that would allow the rapid, reliable and unexpensive detection of aflatoxigenic isolates among a diverse groups of fungi. In order to screen potential aflatoxin-producing fungi on Iranian pistachio samples naturally contaminated with aflatoxin B<sub>1</sub>, we have prepared a specific culture medium known as APA.

## Screening of aflatoxin-producing...

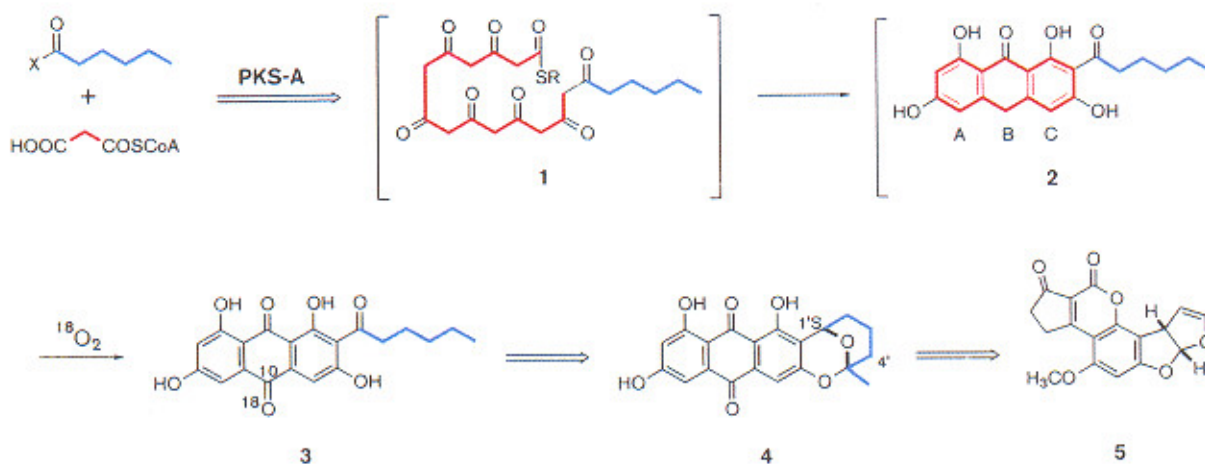


Fig. 1. Polyketide origin of aflatoxin biosynthesis. It is perposed that fatty acid synthase (FAS) enzymes synthesize the hexanoyl starter unit (in blue) to which a polyketide synthase (PKS) homologates malonyl units (in red) to initiate anthrone formation and aflatoxin biosynthesis (4)

1; primary intermediate, 2; anthrone, 3; norsolorinic acid, 4; averufin, 5; aflatoxin B<sub>1</sub>

This medium is made up of ingredients which give blue fluorescence with aflatoxins secreted in the surrounding medium.

## MATERIALS AND METHODS

### Microorganisms

A total of 84 isolates of *A.flavus* Link and one isolate of *A.parasiticus* spere together with other isolates listed in Table 1 were cultured and analysed for their aflatoxin-producing ability. All of the isolates were isolated from samples of pistachio nuts determined to be contaminated with aflatoxin B<sub>1</sub>. Likewise, *A. parasiticus* NRRL 2999, aflatoxin producer and *A. flavus* NRRL 3537 an aflatoxin-nonproducing strain were grown and extracted for aflatoxins as reference control strains.

### Media

Primary isolation of fungal genera and species from pistachio nuts was done using three different common media including; Dichloran-rose bengal chloramphenicol agar (DRCA), Czapek-dox agar (CDA) and Sabouraud dextrose agar (SDA).

One-hundred seeds of each sample were taken, superficially sterilized with a solution of 0.4% sodium hypochlorite for 2 minute and subsequently washed three times with sterile distilled water. These seeds were then placed under aseptic conditions in mentioned culture media (8 seeds for each petridish) and incubated at 28 °C for 3 to 7 days.

A selective medium as *Aspergillus flavus* and *parasiticus* agar (AFPA) was used for rapid detection of *A.flavus* and *A.parasiticus* among the members of the genus *Aspergillus* (19). These species could produce a

persistent bright yellow-orange pigment, near Cadmium Yellow under nonsporulating colonies (Fig. 2) The composition of the medium is given in Table 2. The isolates were cultured on AFPA in plates and incubated for 42-48 hour at 30 °C. Specific pigment is produced as a result of the reaction of aspergillie acid or neoaspergillie acid from the isolates with ferric ammonium citrate of the medium. Final identification of *A.flavus* and *A.parasiticus* as species among the *Aspergilli* were done according to the macroscopic and microscopic criteria (20).

Aflatoxin-producing ability (APA) medium was used in screening the mould isolates for their aflatoxigenicity (21). The composition of the medium is summarized in Table 3. To assay the aflatoxin production, five microliter of spore suspension (approximately 500 conidia) was inoculated at the center of APA medium in a glass petridish and incubated at 28 °C in the dark for 7 to 10 days. Plastic dishes are not suggested because of their interference with fluorescence production. Isolates of *A.flavus* and *A.parasiticus* were also screened for their aflatoxin-producing ability by using the semimicro culture technique with some modifications (22). Cultures were inoculated in 10 ml of sucrose-low salts (SLS) medium on 50 ml erlenmeyer flasks and incubated for 8 days at 28 °C before the extraction process.

### Extraction and assay of aflatoxins

Total content of APA medium (about 20 g) showing blue fluorescence was removed and mixed with 50 ml of distilled water and then homogenized 3 times each time for about 2 minute in a Heidolph DIAX 600 homogenizer. The aqueous slurry was extracted for 10



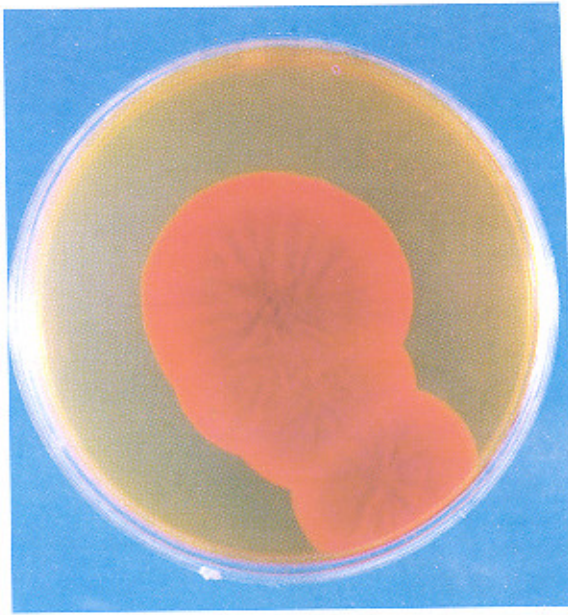


Fig. 2. Intense yellow-orange reverse coloration in colonies of *A.flavus* and *A.parasiticus* on AFPA medium

minute by mixing with 20 ml of chloroform. The mixture was then centrifuged, and the chloroform layer (lower phase) was separated and retained. Following the incubation period required for aflatoxin biosynthesis on SLS medium, flasks were subjected to a very short heat treatment at a temperature of 121 °C to kill the mould. Aflatoxins were extracted from mycelia and media with hot chloroform (55 °C). The chloroform fractions obtained from each culture media were pooled, filtered and concentrated near to dryness using vacuum evaporator apparatus (Heidolph WB 2000). Residues were dissolved in a known volume of chloroform and analysed by TLC technique on 20 × 20 cm silica gel precoated aluminium sheets (E. MERCK). The plates were developed using two different solvent systems known as chloroform-methanol (98:2, vol/vol) or acetone-chloroform-water (12: 88:15, vol/vol/vol). Aflatoxin spots were observed under UV light and then, for quantitative analysis, TLC plates were scanned with a Desaga CD 60 fluorodensitometer by comparison of samples with different dilutions of aflatoxin B<sub>1</sub> reference standard run on the same plates. Visualization of the spots was performed by spraying the plates with 25% sulphuric acid.

## RESULTS

A total of 35 Iranian pistachio nut samples determined to be contaminated with aflatoxin B<sub>1</sub> were examined for the presence of fungal genera and species

specially members of the genus *Aspergillus*. Based on the macroscopic and microscopical criteria, a total of 166 isolates belonging to 8 genera of the fungi were isolated from nuts (Table 1). The frequency of fungal contamination of nuts with genera *Aspergillus*, *Penicillium* and *Alternaria* was greater than other isolates. Table 4 shows the results of the specific blue fluorescence production of selected isolates on APA medium under UV light as a preliminary screening assay of aflatoxin production. The results clearly showed that only 12 out of the 85 *A.flavus* and *A. parasiticus* strains were fluorescent or APA-positive (Fig. 3).

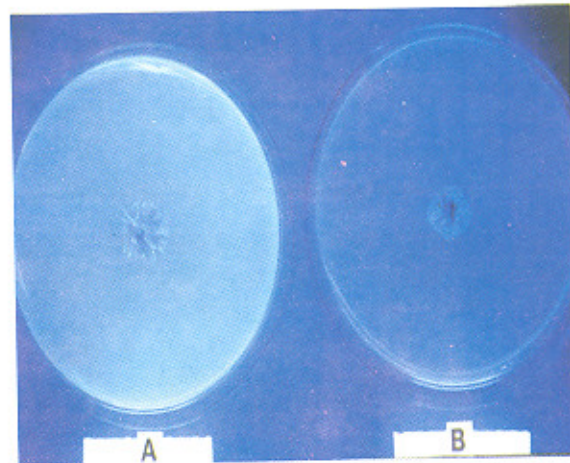


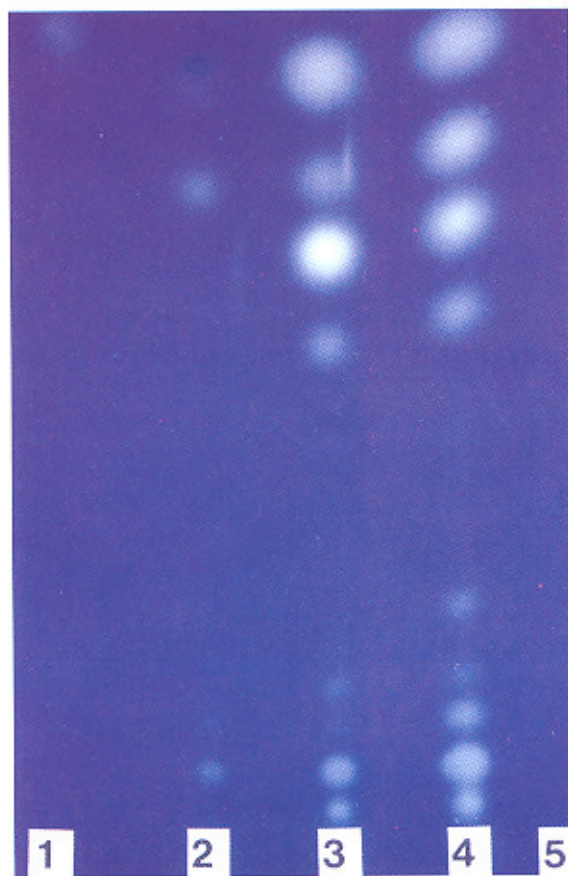
Fig. 3. Colonies of aflatoxin-positive (A) and aflatoxin-negative (B) strains of *A.flavus* and *A.parasiticus* on aflatoxin-producing ability (APA) medium under UV light showing characteristic fluorescence of agar surrounding the aflatoxin-producing strain.

These strains were designated as aflatoxin positive on APA medium after confirmation with complementary analysis. Aflatoxin production of these strains was directly observed on TLC plates (Fig. 4). The pattern of aflatoxin production on TLC plates by *A.flavus* and *A.parasiticus* strains was different. TLC chromatograms showed that four different naturally occurring aflatoxins known as B<sub>1</sub>, B<sub>2</sub>, G<sub>1</sub> and G<sub>2</sub> were produced by *A.parasiticus* strain, whereas toxigenic strains of *A.flavus* produced only B group of aflatoxins (Table 5). The presence of aflatoxin B<sub>1</sub> was confirmed by its R<sub>f</sub> value which was comparable to aflatoxin B<sub>1</sub> standard (Fig. 4). The amount of aflatoxin B<sub>1</sub> produced by *A.parasiticus* strains on APA medium was highest among all of the isolated (3.45-3.71 µg/g medium). It is interesting to note that *A.parasiticus* isolated from pistachio (number 14) can produce even more aflatoxin B<sub>1</sub> than the standard strain (NRRL 2999) on APA medium. A nontoxigenic standard strain of *A.flavus* NRRL 3537 was grown and analysed for aflatoxin



**Table 1.** Frequency of occurrence of principal fungal genera and species in pistachio nuts

Moulds	No. of isolates	No. of positive samples	Percent of contaminated samples
<i>Aspergillus flavus</i>	84	23	65.71
<i>Aspergillus parasiticus</i>	1	1	2.85
<i>Aspergillus niger</i>	15	8	22.85
<i>Aspergillus tamarii</i>	4	3	8.57
<i>Aspergillus ochraceus</i>	1	1	2.85
<i>Aspergillus clavatus</i>	1	1	2.85
<i>Aspergillus</i> spp.	2	2	5.71
<i>Penicillium</i> spp.	18	15	42.85
<i>Fusarium</i> spp.	8	5	14.28
<i>Acremonium</i> spp.	2	2	5.71
<i>Cladosporium</i> spp.	3	3	8.57
<i>Alternaria</i> spp.	12	9	25.71
<i>Rhizopus</i> spp.	9	8	22.85
<i>Mucor</i> spp.	6	5	14.28



**Fig. 4.** TLC analysis of chloroform extracts prepared from the agar surrounding the mould colonies as described in Materials and Methods. The chloroform extracts developed by TLC and photographed by fluorescence. 1; aflatoxin B<sub>1</sub> (standard), 2; *A.flavus* 8, 3; *A.parasiticus* 14, 4; *A.parasiticus* NRRL 2999, 5; *A.flavus* NRRL 3537.

**Table 2.** The composition of *Aspergillus flavus* and *parasiticus* agar (AFPA) medium (19)

Peptone, bacteriological	10 g
Yeast extract	20 g
Ferric ammonium citrate	0.5 g
Chloramphenicol	100 mg
Agar	15 g
Water, distilled	1 litre
Dichloran	2 mg (0.2% in ethanol, 1.0 ml)

a. Initial pH of the medium was 6.2.

**Table 3.** The composition of aflatoxin-producing ability (APA) medium (21)

Distilled water	1 litre
(NH <sub>4</sub> ) H <sub>2</sub> PO <sub>4</sub>	10 g
K <sub>2</sub> HPO <sub>4</sub>	1 g
MgSO <sub>4</sub> · 7H <sub>2</sub> O	0.5 g
KCl	0.5 g
FeSO <sub>4</sub> · 7H <sub>2</sub> O	0.01 g
Sucrose	30 g
HgCl <sub>2</sub>	5 × 10 <sup>-4</sup> M
Corn steep liquor <sup>b</sup>	0.5 g
Agar	20 g

a. pH adjusted to 5.5 with 1N NaOH before the addition of agar.

b. prepared in our laboratory from fresh corn.

**Table 4.** Production of blue fluorescence on APA medium and assay of its correlation with TLC results

Moulds	No. of examined strains	No. of fluorescent strains	No. of aflatoxin producing strains	
			on APA	on SLS
<i>Aspergillus flavus</i>	84	11	11	14
<i>Aspergillus parasiticus</i>	1	1	1	1
<i>Aspergillus niger</i>	5	-	-	-
<i>Aspergillus tamarii</i>	4	-	-	-
<i>Aspergillus ochraceus</i>	1	-	-	-
<i>Aspergillus clavatus</i>	1	-	-	-
<i>Aspergillus</i> spp.	2	-	-	-
<i>Penicillium</i> spp.	4	-	-	-
<i>Fusarium</i> spp.	3	-	-	-
<i>Alternaria</i> spp.	4	-	-	-
<i>Mucor</i> spp.	2	-	-	-
<i>Acremonium</i> spp.	2	-	-	-
<i>Rhizopus</i> spp.	3	-	-	-
<i>Cladosporium</i> spp.	2	-	-	-
<i>F.graminearum</i> <sup>a</sup>	1	-	-	-
<i>T. harizanum</i> <sup>b</sup>	1	-	-	-

a,b: Known toxigenic strains

**Table 5-** Production of aflatoxin by *A.flavus* and *A.parasiticus* isolates on APA and SLS media and assay of its concentration and fluorescent production on APA medium

Isolate number	Aspergillus species	Toxin identified on								Concentration of AFB <sub>1</sub> (µg/g APA medium)	Fluorescence on APA
		APA				SLS					
		B <sub>1</sub>	B <sub>2</sub>	G <sub>1</sub>	G <sub>2</sub>	B <sub>1</sub>	B <sub>2</sub>	G <sub>1</sub>	G <sub>2</sub>		
2999	<i>A.parasiticus</i> NRRL	+	+	+	+	+	+	+	+	3.45	+
3537	<i>A.flavus</i> NRRL									-	-
14	<i>A.parasiticus</i> spcare	+	+	+	+	+	+	+	+	3.71	+
59	<i>A.flavus</i> Link	+	+			+	+			1.49	+
35	<i>A.flavus</i> Link	+				+				1.24	+
24	<i>A.flavus</i> Link	+	+			+	+			1.16	+
7	<i>A.flavus</i> Link	+				+				1.12	+
13	<i>A.flavus</i> Link	+				+				0.86	+
16	<i>A.flavus</i> Link	+				+				0.68	+
75	<i>A.flavus</i> Link	+				+				0.57	+
19	<i>A.flavus</i> Link	+				+				0.51	+
66	<i>A.flavus</i> Link	+				+				0.42	+
2	<i>A.flavus</i> Link	+				+				0.37	+
8	<i>A.flavus</i> Link	+	+			+	+			0.21	+
43	<i>A.flavus</i> Link					+				-	-
31	<i>A.flavus</i> Link					+				-	-
48	<i>A.flavus</i> Link					+	+			-	-

APA : Aflatoxin-producing ability medium

AF: Aflatoxin

SLS : Sucrose-low salts medium

production and used as a negative control strain. This strain and all of the other aflatoxin-nonproducing strains isolated from pistachios did not produce any fluorescence on APA medium (Table 4). In order to further evaluate the UV test results, all of the APA negative strains of *A.flavus* were grown on SLS medium which supports aflatoxin biosynthesis and then analysed for aflatoxin production by TLC. The results showed that only 3 out of the 73 APA-negative strains gain the ability to produce aflatoxin (Table 5).

## DISCUSSION

A wide range of the fungi are broadly distributed in the environment which have the capability to contaminate many agricultural commodities. For toxigenic fungi, this contamination leads to the occurrence of certain mycotoxins in final products. The increasing significance of mycotoxins as potential public health hazards has aroused interest in the examination of certain foods and feeds for the presence and incidence of moulds and their ability to produce mycotoxins. Aflatoxin contamination of food and feedstuffs particularly in a large consignment could be determined using several approaches.

Chromatographic procedures such as HPLC used for the final quantitation of aflatoxins are generally

tedious, expensive and time consuming. In addition, they require careful sampling and solvent extraction steps prior to analysis. Isolation of aflatoxin-producing fungi from suspected foodstuffs and identification of their ability to produce aflatoxins using fast and reliable mycological methods under laboratory conditions could be used as a preliminary screening test prior to chemical analytical methods. One of the considerable advantages of these methods is that early exposure of foodstuffs with aflatoxigenic fungi could be easily determined which indicate the predisposition of these materials to aflatoxin contamination.

In present study, the mycoflora of pistachio nuts with special reference on aflatoxigenic fungi were determined using general and specific culture media. At first, the *Aspergilli* were separated from other fungal genera. Later, strains of *A.flavus* and *A.parasiticus* were identified and then grown on a specific culture medium known as APA medium for evaluation of their aflatoxigenicity. Aflatoxin production was primarily noticed by the change in colour of the medium surrounding colonies as a bright blue fluorescence under UV light. Further experiments based on chromatographic analyses e.g. TLC were carried out to confirm aflatoxin-producing ability of the strains.

Various fungal genera and species were isolated from the samples of pistachio nuts as summarized in Table 1. In addition to the presence of toxigenic

*A.flavus* strains, many other species belonging to the genera *Penicillium* and *Fusarium* isolated in this study could be potentially mycotoxigenic. It has been established that the isolates of *A.flavus* and *A.parasiticus* vary greatly in their ability to aflatoxin production (23-26). However, about one-third (30-40%) of the naturally occurring *A.flavus* strains isolated from agricultural commodities are usually aflatoxigenic (27). In the present study, 17.6% of the isolates were capable of producing aflatoxins out of which approximately 80% had been able to produce specific blue fluorescence on the APA medium under UV light. Under different conditions using other food products, investigators could identify between 30 to 67% of the aflatoxin-producing strains based on their blue fluorescence criteria on the APA medium (28). Fortunately, we did not come across with strains which give false positive results on the APA medium. However, others reported that some fungal metabolites such as asperopterins A or B, flavacol and deoxy-hydroxyaspergillic acid might contribute to blue fluorescence production (29, 30). Furthermore, examined strains other than *A.flavus* and *A.parasiticus* could not produce blue fluorescence on the APA medium as shown in Table 4.

These data clearly indicated the specificity of APA medium to identify only aflatoxin-producing strains among various toxigenic and nontoxigenic fungal genera and species. The possibility of converting low aflatoxin producers so called APA-negative to potential aflatoxin-producing strains on SLS medium which supports aflatoxin biosynthesis was also examined. Among 85 isolates studied, only 3 false negative isolates were identified.

Briefly, blue fluorescence production by aflatoxigenic strains on the APA medium is due to the ingredients known as corn steep liquor and  $HgCl_2$  (21, 31, 32). These compounds may enhance the accumulation of aflatoxin precursors by blocking various enzyme systems (21). It is clear that assay of fluorescence production on APA medium can not facilitate the identification of every aflatoxin-producing isolates but undoubtedly, strong producers will always be discovered. Finally, it is concluded that the observation of blue fluorescence on APA medium provides a simple, reliable and relatively fast way of preliminary screening of a large number of moulds for their aflatoxin producing ability.

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