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The Potential of Aflatoxin Production in the *Aspergillus* Section *Flavi* Isolates of Pistachio in Iran

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ABSTRACT

Introduction: Aflatoxin analysis shows that *Aspergillus* section *Flavi* strains include mixtures of the strains that are highly toxic to humans with high levels of aflatoxins, while some others produce moderate levels of aflatoxins along with nontoxigenic strains. The present study aimed to evaluate the potential of Aflatoxin production in the *Aspergillus* section *Flavi* isolates of pistachio in Iran.

Methods: To determine the aflatoxin-producing abilities of *Aspergillus* section *Flavi* isolates in pistachio orchards, 180 pistachio nut samples were collected, and fungal isolation was performed by *Aspergillus flavus* and parasiticus agar (AFPA) medium. In addition, distinct colony morphology in the coconut agar medium, yeast extract sucrose medium supplemented with methyl-β-cyclodextrin, and sucrose low salt medium were used for the distinguishing and screening of toxigenic and atoxigenic isolates. The toxigenicity and aflatoxins production level of the isolates were assayed via thin layer chromatography.

Results: In total, 120 isolates of various parts of the pistachio-growing areas of *Aspergillus* section *Flavi* were identified by the AFPA, 89.15% of which were able to produce one or several types of aflatoxins, while 10.83% of the isolates had no toxin production. Among the investigated isolates, 14.16% produced aflatoxins such as B1, B2, G1, and G2, while 10.83% of the isolates produced B1, B2, and G1, 34.16% of the isolates produced B1 and B2, and only 30% were able to produce the B1 aflatoxin. However, the aflatoxin production of the toxigenic isolates differed within the ranges of 39-21,548, 37-8,432, 97-2,111, and 31-810 ng/g for aflatoxins B1, B2, G1, and G2, respectively.

Conclusion: According to the results, 13 out of 120 investigated isolates produced no aflatoxins, and the toxigenicity of the other isolates potentially varied from extremely low to high levels.

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Introduction

With 479,000 hectares of orchards, Iran is one of the largest producers of pistachio in the world, and Kerman province is the largest producer with 52% of the total production in Iran and more than 212,000 hectares of orchards. Furthermore, Kerman is considered to be the most important pistachio region in Iran and the world, followed by Razavi Khorasan, Yazd, Fars, Semnan, Qom, Markazi, and Sistan-Baluchestan provinces. Currently, Iran, the United States, Turkey, Greece, Syria, and Afghanistan are the largest suppliers of pistachio in the world (1). Evidently, pistachio is an important agricultural commodity, which requires product preparation considerations for international trade. In general, the contamination of pistachio nuts by aflatoxins is a major challenge in the industry.

Aflatoxins are polyketide products of some *Aspergillus* species, such as *Aspergillus flavus*, *A. parasiticus*, *A. nomius*, *A. bombycis*, *A. pseudotamari*, and *A. ochraceoroseus*, as well as the two *Aspergillus* anamorphs, which are *Emericella venezuelensis* and *E. astellata* (2-4). Aflatoxins are severe food safety issues across the world (5, 6). The contamination of food crops such as peanut, pistachio, maize, and cotton by aflatoxins adversely affects food health, the food industry, and food processing,

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which comply with specific regulations and standards for exports (7-10).

Since the discovery of aflatoxins in the early 1960s, *A. flavus* has been considered the main source of aflatoxin contamination in scientific reports, raising economic concerns (11). In addition, this fungus could invade most food crops and is the common cause of the contamination of peanut, maize, wheat, rice, pistachio, and almond (11, 12). Aflatoxin analysis shows that *Aspergillus* section *Flavi* strains include mixtures of the strains that are highly toxic to humans with high levels of aflatoxins, while some others produce moderate levels of aflatoxins along with nontoxigenic strains (13). Additionally, reports have suggested high levels of phenotypic diversity and strains without toxin production capabilities (14, 15).

According to the literature, aflatoxin production has been observed in 35% of the collected isolates on yeast extract sucrose broth culture from Turkey, 28% from China, 48.4% from various substrate media in India (16). In general, several factors influence aflatoxin production, such as the genetic characteristics of aflatoxin-producing fungus and the physicochemical conditions that affect fungal growth. Mycotoxin production depends not only on species, but also on the fungal strain. Notably, *A. flavus* is not the only strain known to cause toxin production (17). Currently, no control strategies are available to prevent the contamination of maize, cotton, and peanut crops by *A. flavus*, and using multiple strategies simultaneously is considered to produce and maintain healthy, toxin-free crops (12). Furthermore, several strategies have been suggested to reduce the damages of toxin-producing fungus before and after crop harvesting, such as chemical, physical, and biological control. In this regard, the most promising pre-harvest control strategy is the use of non-aflatoxigenic (atoxigenic) *A. flavus* strains. Therefore, the toxicity assessment of the isolates obtained from the pistachio orchards of every region is essential to the identification of atoxigenic native isolates for use in biological control.

In the studies conducted by Fani (18, 19), the distribution of the aflatoxigenic strains of *A. flavus* in pistachio-growing areas was investigated. Atoxigenic isolates were collected from various regions of the country with the frequency of 6.2-25%. Among 524 *A. flavus* isolates, 53 and 10 isolates of pistachio nut and soil were atoxigenic, respectively. Furthermore, several studies have indicated that the type and level of aflatoxin production significantly differ in the *A. flavus* isolates collected from agricultural crops (20-24). In Argentina, Magnoli (25) determined the aflatoxin production capacity by the *Aspergillus* section *Flavi* isolated from poultry feeds, and the obtained results showed the potential for mycotoxin production by *Aspergillus* section *Flavi* and *Penicillium* spp.

The present study aimed to investigate the level of pistachio nut contamination by *Aspergillus* section *Flavi* and determine the toxigenicity of the aflatoxin-producing isolates obtained from the major pistachio-growing areas in Iran.

Materials and Methods

Sampling

In this study, isolates were obtained from the processing terminals of the pistachio-growing areas in Iran, including Kerman, Qazvin, Razavi Khorasan, Markazi, Yazd, Qom, Fars, Sistan-Balouchistan, and Semnan provinces. Samples were collected in the last stage of pistachio processing from the terminals. In accordance with our sample preparation method, 10 samples weighing 50 grams were collected from different stages of the terminals separately and mixed to form a complex sample of 500 grams, which resulted in 180 main samples. Notably, the samples were obtained from 35 terminals in different provinces (Table 1).

Isolation

Differential *Aspergillus flavus* and *Parasiticus* agar (AFPA) medium was used for the monitoring and detection of the *Aspergillus* section *Flavi* strains. Ideally, the selective medium picked the two species of *A. flavus* and *A. parasiticus* based on the formation of a bright orange color on the reverse side of the growing colony (26). Since the distribution of aflatoxins in the samples was not homogenous and evaluating *Aspergillus* section *Flavi* contamination level required homogenous samples, the pistachio samples were initially milled and inoculated in the AFPA medium by

serial dilution in order to isolate the strains. Following that, 10 grams of the ground pistachio nuts of each sample was added to 90 milliliters of 0.1% peptone salt solution and shaken with the contents for 20-40 minutes. Afterwards, 0.1 milliliter of 10^{-1}, 10^{-2} dilutions was spread on the AFPA plate. Each petri dish was incubated at the temperature of 28°C for 2-3 days, and the single isolated pure *Aspergillus* section *Flavi* colonies were identified, calculated, and purified. The contamination rate of each sample was compared by counting the *Aspergillus* section *Flavi* colonies. The AFPA medium could also prevent the spore formation of the colonies belonging to the section *Flavi* species, and colony detection and enumeration was generally easy owing to the recognizable and visible fungal colonies.

Aflatoxin Production Potential of Aspergillus Section Flavi Isolates

After the isolation of the strains from the contaminated pistachio, the toxigenic/atoxigenic strains were screened using fluorescence detection (FD) assays, ammonia vapor (AV) assays, and the color change liquid medium method. In addition, thin-layer chromatography (TLC) was used for aflatoxin quantification.

Fluorescence Detection (FD) Assays

The basic principle for FD is the diffusion of toxins into the media (27, 28). For the FD analyses, the coconut agar medium (CAM) and YES-MβC media were used.

- Coconut Agar Medium (CAM)

The isolates were grown on the coconut extract agar medium at the temperature of 28°C for 72 hours and kept in the dark. The detection and quantification of toxigenicity were performed by producing a fluorescent halo around the colonies after three days. The presence (aflatoxin production) or absence (no aflatoxins production) of a characteristic blue fluorescence in the agar surrounding the colonies was visualized under UV light (365 nm) via incubation using a transilluminator (UVP, Upland, CA 91786, USA), which was exposed to UV light at 365 nanometers.

-Yeast Extract Sucrose (YES) Medium Modified with Methyl-β-Cyclodextrin

One and a half grams of methyl-beta-cyclodextrin (C4555, Sigma-Aldrich, Germany) was dissolved in sterile distilled water, and the solution was directly filtered through a 22-micrometer filter and added to 500 milliliters of sterile YES culture medium (sucrose: 100 g, yeast extract: 10 g, agar: 10 g) before pouring into petri dishes (27). The isolates were grown on the modified medium at the temperature of 28°C for three days and kept in the dark. At the next stage, the toxigenicity of the isolates was investigated using the previously described method.

Ammonia Vapor (AV) Assays

To distinguish between the toxigenic and atoxigenic isolates, the YES medium was exposed to AV, so that the color of the colonies would change to pink or red after three days (18). Through this method, no color change of colonies to pink or red proved atoxigenicity of the isolates (18). In this method, the initial screening of the isolates was carried out, and the toxigenic and atoxigenic strains were distinguished.

Color Change in Sucrose Low Salt (SLS) Medium

The color change sucrose low salt (SLS) method was another approach used to identify the toxigenic and atoxigenic isolates in the present study (30). For toxigenicity identification, the fungal spore suspension of the isolates was initially prepared. Following that, $6.5x10^6$-$7x10^6$ spores per milliliter was inoculated to 50 milliliters of the liquid medium (SLS) in an Erlenmeyer flask and preserved at the temperature of 28°C for one week.

Thin-layer Chromatography (TLC) Assay

After the initial screening of the toxigenic isolates by a culture medium and isolate selection, the fungal isolates were inoculated on rice flour to assay the toxigenicity of the isolates, and the toxigenic level of the isolates on the natural substrate (i.e., rice flour) was estimated. In general, the spore suspension of the isolates was prepared, and one milliliter of the fungal spore (10^3 spore/ml) was inoculated on 22 grams of rice flour and preserved at the temperature of 28°C for one week (25). At the next stage, the aflatoxins of the rice medium samples were extracted using chloroform and methanol. Finally, the toxigenicity test was carried out by TLC as a reference method for the confirmation of cultural methods (7). In addition, the plates were developed in

chloroform: acetone (9:1 v/v) and visualized under UV light (365 nm). The aflatoxins were directly quantified on the TLC plates using a scanning densitometer (TLC Scanner 3; CAMAG Scientific Inc., Wilmington, NC).

Table 1. The number of *Aspergillus* section *Flavi* isolates in each province

Province	Number of terminals	Number of samples	Number of isolates	Toxigenic	Atoxigenic	Atoxigenic/toxigenic ratio
Qazvin	3	27	22	19	3	13.6
Kerman	7	31	18	17	1	5.5
Semnan	5	25	15	12	3	20
Fars	3	24	13	12	1	7.7
Sistan-Balouchistan	2	16	12	10	2	16.7
Qom	3	19	12	10	2	16.7
Khorasan Razavi	6	15	11	10	1	9
Yazd	4	14	10	9	1	10
Markazi	2	9	7	6	1	14.3
Total	35	180	120	105	15	12.5

Results

Aflatoxin Production of the Aspergillus Section Flavi Isolates

In total, 120 isolates of various parts of the pistachio-growing areas belonging to *Aspergillus* section *Flavi* were identified by AFPA (Table 2). Out of 120 *Aspergillus* section *Flavi* isolates, 18, 20, 22, and 23 isolates were atoxigenic based on the absence of colony color change upon contact with AV (Figure 1), fluorescence detection, and color change of the liquid medium to yellow using the YES, CAM, YES medium modified with methyl-β-cyclodextrin (Figure 2), and SLS media, respectively (Table 1).

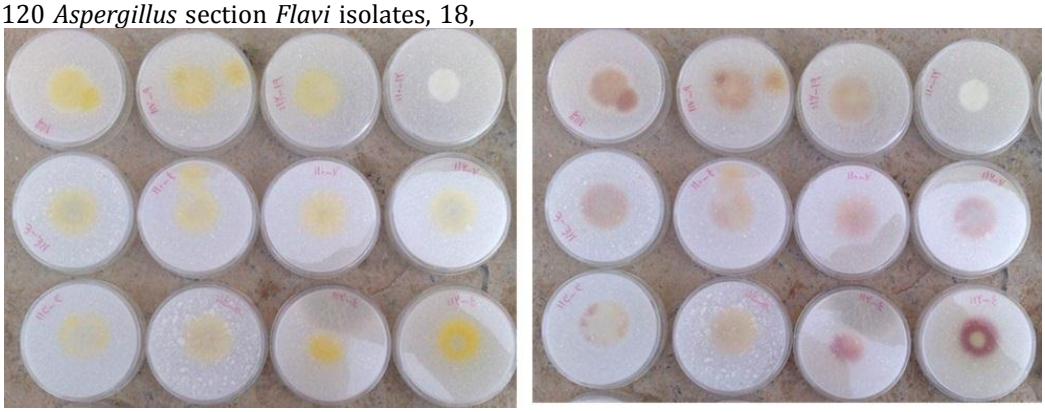

Figure 1. The color of the colony reverse of toxigenic isolates of *Aspergillus* section *flavi* grown on CAM after 3 days incubation at 28°C, colonies at left side and colonies at right side are shown before and after exposure to ammonia vapor, respectively

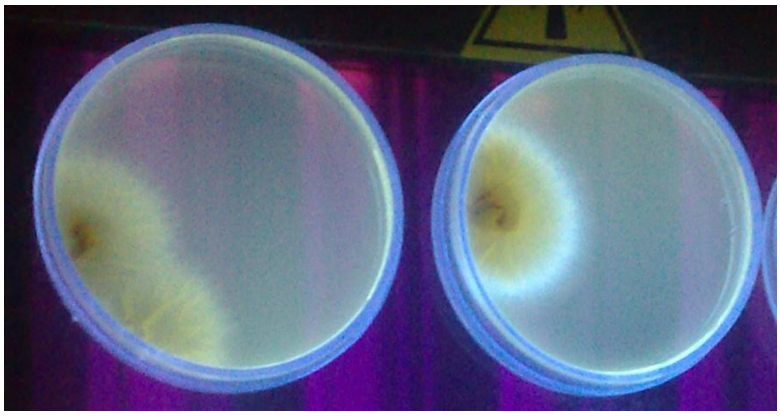

Figure 2. Colonies of nontoxigenic (left) and toxigenic (right) strains observed under UV light (365 nm). Strains were cultivated in YES *medium modified with methyl-β-cyclodextrin* and photographed on the third day of incubation at 28°C

The TLC assays showed that only 13 strains were aflatoxigenic (tables 1 & 2). On the other word, the frequency of the atoxigenic isolates obtained from three cultural media in comparison to 10.83% of the total isolates indicated that the false negative in the detection of the atoxigenic isolates was approximately 4.17-8.33%.

Table 2. *Aspergillus* section *Flavi* isolates characteristics obtained from pistachio nut of different provinces

No.	Isolate	Location (Province)	Aflatoxins (ng/g)				No.	Isolate	Location (Province)	Aflatoxins (ng/g)			
			B1	B2	G1	G2				B1	B2	G1	G2
1	SEP12	Semnan	1852	117	ND	ND	61	FAP72	Fars	157	ND	ND	ND
2	SEP13	Semnan	2354	214	ND	ND	62	FAP73	Fars	15193	1657	1145	293
3	SEP14	Semnan	1357	124	ND	ND	63	FAP74	Fars	1832	ND	ND	ND
4	SEP15	Semnan	1087	67	ND	ND	64	FAP75	Fars	ND	ND	ND	ND
5	SEP16	Semnan	1733	ND	ND	ND	65	FAP76	Fars	465	102	ND	ND
6	SEP17	Semnan	264	ND	ND	ND	66	FAP77	Fars	812	ND	ND	ND
7	SEP18	Semnan	6723	ND	ND	ND	67	FAP78	Fars	1247	135	ND	ND
8	SEP19	Semnan	ND	ND	ND	ND	68	GHP79	Ghazvin	2132	2041	2111	810
9	SEP20	Semnan	294	ND	ND	ND	69	GHP80	Ghazvin	548	ND	ND	ND
10	SEP21	Semnan	1231	124	141	ND	70	GHP81	Ghazvin	ND	ND	ND	ND
11	SEP22	Semnan	2385	117	ND	ND	71	GHP82	Ghazvin	932	ND	ND	ND
12	SEP23	Semnan	ND	ND	ND	ND	72	GHP83	Ghazvin	51	ND	ND	ND
13	SEP24	Semnan	9547	1354	210	ND	73	GHP84	Ghazvin	20712	2152	720	190
14	SEP25	Semnan	8547	1054	284	ND	74	GHP85	Ghazvin	3721	292	576	104
15	SEP26	Semnan	ND	ND	ND	ND	75	GHP86	Ghazvin	ND	ND	ND	ND
16	KHP27	Khorasan Razavi	12547	1235	241	ND	76	GHP87	Ghazvin	871	ND	ND	ND
17	KHP28	Khorasan Razavi	ND	ND	ND	ND	77	GHP88	Ghazvin	2465	354	ND	ND
18	KHP29	Khorasan Razavi	924	90	97	ND	78	GHP89	Ghazvin	354	67	ND	ND
19	KHP30	Khorasan Razavi	39	ND	ND	ND	79	GHP90	Ghazvin	5487	532	652	121
20	KHP31	Khorasan Razavi	9512	981	172	ND	80	GHP91	Ghazvin	584	ND	ND	ND
21	KHP32	Khorasan Razavi	1780	84	ND	ND	81	GHP92	Ghazvin	1157	155	332	101
22	KHP33	Khorasan Razavi	3054	274	ND	ND	82	GHP93	Ghazvin	ND	ND	ND	ND
23	KHP34	Khorasan Razavi	4287	328	ND	ND	83	GHP94	Ghazvin	658	ND	ND	ND
24	KHP35	Khorasan Razavi	697	82	196	31	84	GHP95	Ghazvin	164	37	ND	ND
25	KHP36	Khorasan Razavi	4287	241	514	105	85	GHP96	Ghazvin	362	94	ND	ND
26	KHP37	Khorasan Razavi	2027	179	168	ND	86	GHP97	Ghazvin	215	84	ND	ND
27	KEP38	Kerman	9679	1130	188	ND	87	GHP98	Ghazvin	1050	ND	ND	ND
28	KEP39	Kerman	8517	1432	193	ND	88	GHP99	Ghazvin	1987	ND	ND	ND
29	KEP40	Kerman	875	108	ND	ND	89	GHP100	Ghazvin	1254	72	ND	ND
30	KEP41	Kerman	6397	ND	ND	ND	90	MAP101	Markazi	1347	198	245	81
31	KEP42	Kerman	13612	1547	275	ND	91	MAP102	Markazi	1782	105	ND	ND
32	KEP43	Kerman	1093	ND	ND	ND	92	MAP103	Markazi	6524	532	ND	ND
33	KEP44	Kerman	1475	81	ND	ND	93	MAP104	Markazi	ND	ND	ND	ND
34	KEP45	Kerman	741	83	287	51	94	MAP105	Markazi	357	ND	ND	ND
35	KEP46	Kerman	ND	ND	ND	ND	95	MAP106	Markazi	2187	298	285	122
36	KEP47	Kerman	458	64	ND	ND	96	MAP107	Markazi	7832	608	ND	ND
37	KEP48	Kerman	154	ND	ND	ND	97	SIP108	Sistan-Balouchistan	1892	ND	ND	ND
38	KEP49	Kerman	21548	8432	491	241	98	SIP109	Sistan-Balouchistan	518	93	ND	ND
39	KEP50	Kerman	3524	335	ND	ND	99	SIP110	Sistan-Balouchistan	ND	ND	ND	ND
40	KEP51	Kerman	4587	465	ND	ND	100	SIP111	Sistan-Balouchistan	2125	ND	ND	ND
41	KEP52	Kerman	18	ND	ND	ND	101	SIP112	Sistan-Balouchistan	1125	71	ND	ND
42	KEP53	Kerman	4325	241	ND	ND	102	SIP113	Sistan-Balouchistan	1532	214	270	91
43	KEP54	Kerman	428	192	ND	ND	103	SIP114	Sistan-Balouchistan	577	72	ND	ND
44	KEP55	Kerman	691	ND	ND	ND	104	SIP115	Sistan-Balouchistan	513	ND	ND	ND
45	YAP56	Yazd	1958	187	ND	ND	105	SIP116	Sistan-Balouchistan	1530	210	271	93
46	YAP57	Yazd	1102	111	162	ND	106	SIP117	Sistan-Balouchistan	ND	ND	ND	ND
47	YAP58	Yazd	872	91	ND	ND	107	SIP118	Sistan-Balouchistan	13127	1812	294	ND
48	YAP59	Yazd	631	94	342	82	108	SIP119	Sistan-Balouchistan	514	86	ND	ND
49	YAP60	Yazd	ND	ND	ND	ND	109	QOP120	Qom	7824	632	ND	ND
50	YAP61	Yazd	1238	ND	ND	ND	110	QOP121	Qom	298	ND	ND	ND
51	YAP62	Yazd	887	273	ND	ND	111	QOP122	Qom	633	97	ND	ND
52	YAP63	Yazd	67	ND	ND	ND	112	QOP123	Qom	5421	409	ND	ND
53	YAP64	Yazd	487	ND	ND	ND	113	QOP124	Qom	ND	ND	ND	ND
54	YAP65	Yazd	349	ND	ND	ND	114	QOP125	Qom	1254	298	157	ND
55	FAP66	Fars	1250	82	ND	ND	115	QOP126	Qom	193	ND	ND	ND
56	FAP67	Fars	4521	657	425	107	116	QOP127	Qom	437	86	ND	ND
57	FAP68	Fars	912	ND	ND	ND	117	QOP128	Qom	1233	153	219	75
58	FAP69	Fars	542	112	ND	ND	118	QOP129	Qom	1588	169	ND	ND
59	FAP70	Fars	1547	284	ND	ND	119	QOP130	Qom	1457	143	ND	ND
60	FAP71	Fars	91	ND	ND	ND	120	QOP131	Qom	1235	ND	ND	ND

ND: Not Detected

The evaluation of the aflatoxigenicity of the *Aspergillus* section *Flavi* isolates obtained from the pistachios grown in various regions of Iran indicated that the isolates had variable toxin production (Table2). Out of 120 studied *Aspergillus* section *Flavi* isolates, 107 isolates (89.15%) produced one or several types of aflatoxins (B1, B2, G1, and G2), while 13 isolates (10.83%) were unable to produce any aflatoxins. In addition, the toxigenicity of the other isolates potentially varied from extremely low to high. Among the toxigenic isolates,

14.16% (n=17) produced four types of aflatoxins (B1, B2, G1 and G2), 10.83% (n=13) produced B1, B2 and G1, 34.16% (n=41) produced B1 and B2, and 30% (n=36) only produced only B1 (Table 2).

Among the *Aspergillus* section *Flavi* isolates, toxigenicity varied from high to low toxicity; such examples were B1 aflatoxin production (39-21,548 ng/g), B2 aflatoxin production (37-8,432 ng/g), G1 aflatoxin production (97-2,111 ng/g), and G2 aflatoxin production (31-810 ng/g) (Table 2). However, the results of the present study indicated that belonging to *Aspergillus* section *Flavi* species is not the only reason for aflatoxigenicity. Out of 120 investigated isolates, 13 isolates produced no aflatoxins, and the toxigenicity of the other isolates potentially varied from extremely low to high.

Based on the results of the SLS method, 23 isolates (19.6%) were atoxigenic. In the fluorescence detection, 22 (18.33%) and 20 isolates (16.66%) were initially scored as atoxigenic based on the YES-MβC and CAM, respectively. In contrast, fewer isolates were identified as atoxigenic using the AV assays. Accordingly, only 18% of the isolates (n=15) were initially considered atoxigenic based on the absence of color change upon contact with the AV (Table 3). In general, the accuracy of the applied methods declined in the YES-AV, PDA-AV, CAM-FD, and YES-MβC-FD, respectively. In addition, the frequency of the false negatives ranged from 4.7% (AV using YES) to 10% (SLS), indicating that most of the methods overestimated the frequency of the atoxigenic isolates.

Table 3. Frequency of toxigenic (+) and Atoxigenic (-) *Aspergillus* section *flavi* isolates using cultural and TLC assays

Group	Number of isolates	Source	SLS[1]	FD[2] on		AV[5] on YES[6]	TLC[7]	Atoxigenic (%)	Method Accuracy
				YES-MβC[3]	CAM[4]				
1	107	Nut	+	+	+	+	+		
2	23	Nut	-	+	+	+	+	19.16	90
3	22	Nut	-	-	+	+	+	18.33	91.5
4	20	Nut	-	-	-	+	+	16.66	93.4
5	18	Nut	-	-	-	-	+	15	95.3
6	13	Nut	-	-	-	-	-	10.83	100

[1] SLS = Sucrose low salt medium
[2] FD = Fluorescence detection assay
[3] YES-MβC= Yeast extract sucrose medium amended with 0.3% methyl β-cyclodextrin
[4] CAM = Coconut agar medium
[5] AV = Ammonium Vapor assay
[6] YES= Yeast extract sucrose medium
[7] TLC = Thin layer chromatography

Discussion

Mycotoxins are among the most important concerns regarding food safety and human health, particularly aflatoxins in agricultural products and nuts, which have led to imposing strict rules, regulations, and costs for mycotoxin management from production until consumption. Some *Aspergillus* species of the subgenus *Circumdati* section *Flavi* have the ability to produce aflatoxins, and the potential has urged extensive research regarding fungal secondary metabolites (34). These strains are known as opportunistic pathogens, and their ability to produce aflatoxins in numerous food products (especially nuts) shows their great importance and obvious impact on agricultural products. Furthermore, investigating the species of the *Flavi* section has added to the current body of knowledge regarding the evaluation of these strains in other fields, such as medicine, ecology, genomics, entomology, plant genetics, toxicology, economy, mycology, plant pathology, and biochemistry (31-34).

An effective pre-harvest control strategy for agricultural products involves the use of *A. flavus* atoxigenic strains as several studies have been focused on their ability to modify natural *A. flavus* populations, interfere with aflatoxin biosynthesis or competitively eliminate aflatoxin producers in crops. Aflatoxin production varies among different isolates for several reasons, the most important of which is the genetic diversity of isolates as stated in the previous studies in this regard (19-24, 20, 21, 36, 37). For instance, Mirabolfathy (38) evaluated the correlation between sclerotia formation and aflatoxin production in 120 *A. flavus* isolates, reporting that 88.5% of the

sclerotia-producing isolates produced about ten times more aflatoxins compared to the non-sclerotia-producing isolates. Another study in this regard was conducted by Zamani (39) on the structure of *A. flavus* population to investigate the aflatoxin-producing abilities. According to the findings, the vegetative compatibility groups (VCGs) and sclerotic-producing isolates were diversified, with 63% producing aflatoxins and 36.58% identified as atoxigenic isolates. However, no significant correlations were observed between the geographical origins, hosts, VCGs, and sclerotium-producing and aflatoxin-producing abilities.

In another research, Alibakhshi (40) discussed the effect of anastomosis on the transfer of the genetic material between toxigenic and atoxigenic *A. flavus* isolates, which were obtained from pistachios. According to the ELISA test results, 80% of the isolates produced aflatoxins, and 19% were atoxigenic. In addition, Mohammadi (21) carried out an investigation in Fars and Kerman provinces (Iran), isolating 13 species of *Aspergillus* obtained from the soil of numerous crops, among which *A. carbonarius* and *A. oryzae* were detected as two new species for the fungal flora of Iran. Furthermore, more than half of the *A. flavus* samples were identified as aflatoxigenic strains. In the studies performed by Fani (23, 24), the authors investigated the distribution of aflatoxigenic *A. flavus* strains of pistachio-growing areas. In the mentioned study, the atoxigenic isolates were collected from various regions of the country with the frequency of 6.2-25%. According to the obtained results, 53 and 10 out of 524 *A. flavus* isolates from pistachio nut and soil were atoxigenic. In addition, many researchers have reported that the type and level of aflatoxin production vary in the *A. flavus* isolates collected from agricultural crops (20-24).

The results of the present study confirmed that among the *Aspergillus* section *Flavi* isolates, a small percentage of the strains were atoxigenic (10.83%), which demonstrated the atoxigenic potential of the isolated strains in the biological control of aflatoxigenic strains, as well as the necessity of the screening and isolation of atoxigenic strains despite the high detection costs. The mentioned study also assessed the toxigenicity of *Aspergillus* section *Flavi* isolates in order to distinguish the toxigenic and atoxigenic strains. The initial screening method was primarily performed to isolate the strains using rice flour by culture media, including the coconut agar medium, YES medium modified with methyl-β-cyclodextrin, YES medium with AV, and SLS, while the toxigenicity of the isolates was estimated using the TLC method.

In the current research, the comparison of various methods for the screening of the atoxigenic isolates yielded different results, while the accuracy of the TLC was reported to be higher than the culture media. Among various culture media, better results regarding the isolation of the toxigenic strains were observed with the colony color change to pink or red through exposure to the AV, YES medium, coconut extract agar media, YES medium modified with methyl-β-cyclodextrin, and SLS. Furthermore, the obtained results indicated that for the final detection and confirmation of the non-toxigenicity of the *Aspergillus* section *Flavi* isolates, reference methods such as the TLC and high-performance liquid chromatography (HPLC) are required since the cultural methods estimated atoxigenicity more accurately (11). In addition, these methods are simple, cost-efficient, and rapid with high availability and propriety for use in initial screening. On the other hand, one of the challenges associated with media such as the YES modified with methyl-β-cyclodextrin and AV for determining the toxigenicity of *Aspergillus* section *Flavi* isolates is the inability to identify the isolates with low toxigenicity and their identification as atoxigenic isolates. In the current research, the described media indicated the higher frequency of the atoxigenic isolates, which was false. Therefore, the use of methods with higher accuracy in this regard in addition to the culture media used for initial monitoring seems essential. With respect to the presence of atoxigenic isolates in the orchards, the higher frequency of these isolates was confirmed in Semnan province compared to the other studied areas.

According to the results of the present study, medias such as the CAM and YES medium modified with methyl-β-cyclodextrin and AV are more appropriate than laboratory analytical techniques (e.g., TLC and HPLC) for the initial screening of isolates due to the high costs of

laboratory techniques. Nevertheless, accurate laboratory methods (e.g., HPLC, ELISA, and TLC) are considered affordable and convenient for the final recognition of toxigenic isolates.

In general, biological control is an effective strategy for pre-harvesting in agricultural and horticultural products, and several methods have been used for this purpose, especially application of atoxigenic *A. flavus* isolates for aflatoxin mitigation. Evidently, success in the biological control of toxigenic fungus depends on the native isolates of the region in most of the cases. As a result, it is essential to identify the aflatoxigenicity and population varieties of *A. flavus* regarding aflatoxin production in the growth areas of pistachio, while the isolation of the atoxigenic isolates of each region is also important for managing the population of *A. flavus* toxigenic strains.

In the present study, 13 native atoxigenic isolates from Damghan, Rafsanjan, Faizabad, Yazd, and Sabzevar regions were obtained from the orchards for a complete research. Considering the increasing development of pistachios in various regions of Iran, it is recommended that the aflatoxigenicity of the fungal isolates in the other pistachio-growing regions be investigated.

In the current research, the evaluation of the isolates obtained from the pistachio nut samples of various terminals indicated the highest rate of toxin production in the isolates of Kerman province. On the other hand, the atoxigenic/toxigenic ratio in Kerman province was 5:5, which was the lowest compared to the other provinces. This could be attributed to the ecological dominance of toxigenic isolates over nontoxigenic isolates in this region. The other influential factors in this regard could be environmental factors, genetics, fungal evolution, and microbial population. The section *Flavi* isolates of Qom and Sistan-Balouchistan provinces had the lowest rates of toxigenic isolates compared to the other provinces, and the rate of toxigenic isolates in these provinces was trice lower than Kerman province. This could be due to the relatively low area under cultivation and new orchards, which led to the lower ratio of the toxin-producing isolates, compared to a province such as Kerman with a cultivated area of approximately 300,000 hectares.

Conclusion

In this study, 35 pistachio processing terminals were sampled in the major pistachio-growing areas in Iran. In total, 180 pistachio nut samples were collected, and 120 *Aspergillus* section *Flavi* isolates were obtained, 13 of which were nontoxigenic based on the cultural and analytical assays. In the aflatoxin-producing isolates, toxigenicity potentially varied from extremely low to high. Therefore, it could be concluded that cost-efficient methods for the detection of aflatoxin production in cultures may be appropriate in case of limited resources. Based on the TLC assay as an analytical reference method, the accuracy of the cultural methods declined as YES-AV, PDA-AV, CAM-FD, and YES-MβC-FD, respectively.

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Conflicts of interest

None declared.

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