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Correlation of *Aspergillus flavus* Growth and Aflatoxin Production with Sugar and Lipid Content among Pistachio Cultivars

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ABSTRACT

Aflatoxin is an important fungal contaminant in various crops. The substrate and its compounds are involved in the growth of fungus and the production of toxins. In order to investigate correlation between sugar and lipid content as the major chemical composition of pistachio kernels with *Aspergillus flavus* growth and aflatoxin production, the total sugar content was extracted by phenol-sulphoric method and estimated by spectrophotometry. The lipid was also extracted by Soxhlet method. A toxigenic *A. flavus* strain was used for the study of the fungal growth and aflatoxin B_1 production. For that, 20 gram of kernels of different pistachio cultivars were inoculated with 1 ml of fungal spore suspension (2×10^6 spores/ml) in a completely randomized design with three replications. Eight days after inoculation, the average percent of fungal growth and colonization of *A. flavus* were calculated. The aflatoxin produced in inoculated pistachios was extracted by Best Foods (BF) method and measured by thin layer chromatography (TLC). The results showed that there was no significant difference in the amount of sugar content in different cultivars of pistachios kernel but the amount of total lipid content was different ($P\leq0.05$). Results of statistical analysis showed that there was no significant correlation between sugar content with fungal growth and aflatoxin production in pistachio cultivars as well as lipid percentage and aflatoxin B1 production. Therefore, to find sources of resistance to *A. flavus* in commercial pistachio cultivars in aflatoxin management programs, these two factors cannot be used as a standard scale of resistance.

Introduction

Twenty percent of the world's food products are contaminated with mycotoxins annually. Damage from aflatoxins in the destruction of food and agricultural products in the United States is estimated at more than $ 100 million per year (Ehrlich, 2014). In Africa, more than $ 750 million is the annual cost of aflatoxin contamination (Gbashi *et al*., 2018). Pistachio with a production equivalent to 200,000 tons with high export value, after oil is the most important source of foreign exchange earnings in Iran (Hosseini

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et al., 2022). Aflatoxin contamination has been the main challenge of its export since 1971 (Danesh *et al*., 1979). Contamination of pistachios with *Aspergillus* species and aflatoxin has placed restrictions on pistachio exports (Fani *et al*., 2014a; Salehi *et al*., 2018; Moghadam *et al*., 2020). The most important factor in the production of aflatoxins is isolates belonging to several species of *Aspergillus*, especially *A. flavus*. The genus *Aspergillus* includes more than 200 species. The different species of this fungus have a wide range of distribution and are found from the Polar Regions to the tropical warm regions. Species of the genus *Aspergillus* are able to secrete numerous enzymes and, therefore, can grow on different substrate. In fact, it is difficult to find a nutrient medium that contains some organic matter and a small amount of moisture that *Aspergillus* species cannot grow on. Thus, *Aspergillus* species affect human life in different ways (Amaike and Keller, 2011). More than 13 species of *Aspergillus* are capable of producing aflatoxins B1, B2, G1 and G2. The most important aflatoxigenic species is *A. flavus* Link. This species adapts well to difficult environmental conditions and, therefore, has a global distribution and is more important than other species. *A. flavus* spends most of its life cycle in soil, plant or animal organic matter as saprophyte. Mycelia are the main structure of microorganisms in the soil and its long-term survival is possible with the production of sclerotium. In addition to aflatoxins, *A. flavus* can produce 14 other types of mycotoxins (Pourhosseini *et al*., 2020). Aflatoxin biosynthesis occurs under certain environmental conditions in terms of light, temperature, acidity (pH), nitrogen source, carbon source and metals. Understanding the relationship between these factors and aflatoxin biosynthesis is critical in determining the role of aflatoxin in fungal ecology (Mahbobinejhad *et al.*, 2019). Acidic conditions are more favorable for aflatoxin production than alkaline conditions, and transcripts of the aflatoxin gene group in alkaline conditions are more negatively regulated than in acidic conditions. Metals, especially zinc, are important factors in the production of aflatoxins. A mixture of copper, iron and zinc increases gene expression and aflatoxin production. The optimum temperature for aflatoxin production is between 28 and 30°C, and aflatoxin production decreases as the temperature approaches the optimum temperature for fungal growth (37°C) (Amaike and Keller, 2011). More than 30 genes are clusters approximately 75 kb on chromosome 7 of the fungus, involving a significant number of enzymatic pathways and in a very complex process of aflatoxin production (Chang *et al*., 2005; Fani *et al*., 2011).

Different methods have been recommended to mitigate the contamination of different crops with *Aspergillus* or Aflatoxin, such as cultural, mechanical, physical and biological methods, each of which have advantages and disadvantages depending on the place, time, type of product, applicability and efficiency. (Barkai-Golan and Paster, 2011; Moradi and Hokmabadi, 2011; Fani *et al*., 2014; Moradi and Fani, 2018; Rouhani *et al*., 2018; Moradi *et al*., 2020).

Thus, it is obvious that all different aspects of *A. flavus* and aflatoxin must be studied and considered in a comprehensive and integrated manner. Given the fact that sugar content of pistachio kernels are among their most important chemical compositions that make the environment suitable for fungal invasions, and are in fact a very important factor influencing fungal growth and aflatoxin production, and since difference in chemical composition of various cultivars of the same product may form the basis of its resistance or susceptibility against *A. flavus* and aflatoxin, this paper has been devoted to measuring sugar content of kernels of pistachio cultivars and studying the likely correlation between their levels and the growth of *A. flavus* and aflatoxin B_1 production.

Many studies have been done on the role and relationship of phenolic compounds, sugars, proteins, fats and fatty acids with fungal growth and aflatoxin production in various products including peanuts, almonds, corn and legumes (Samapundo *et al*., 2007; Latha *et al*., 2007; Nigam *et al*., 2009; Cary *et al*.,

2013; Pizzolitto *et al*., 2015). Premlata *et al*. (1990) measured the levels of sugar, protein and phenol in 38 cultivars of legumes and investigated the role of these compounds in the susceptibility or resistance of cultivars to *A. flavus* growth and aflatoxin production. The results of this study showed that the amount of protein and phenol in resistant cultivars was much higher than susceptible cultivars. While the total sugar content in susceptible cultivars was higher than resistant cultivars (Premlata *et al*., 1990).

The aim of this study was to measure the sugar and lipid content of pistachio kernels of different cultivars (as the most important chemical compounds in pistachio kernels) and to study the possible relationship between aflatoxin B1 production and sugar and lipid levels.

Materials and Methods

Measuring sugar content of pistachio cultivars

To study the possible correlation between sugar contents of kernels of different pistachio cultivars (Akbari, Abbasali, Shahpasand, Kallehbozi, FAS-13-73, Kalleghoochi, Ahmadaghaee, Ovhadi and Khanjari) and *A. flavus* growth and aflatoxin B_1 production, the amount of sugar contained in different pistachio cultivars were measured. The total sugar content in pistachio kernels was extracted by phenol-sulphoric method and estimated by spectrophotometry (Mirrokni *et al*., 2014). The assay steps were as follows:

1-To remove moisture, pistachio kernels were dried in an oven at 48-70°C for 48 hours and then ground.

2-Ten ml of 80% ethanol on 0.1 g of pistachio kernels of each cultivar was added to a jug flask and the balloons were connected to refrigerant. Three replications were considered for each cultivar.

3-The balloons were placed in a container containing boiling water for 15 minutes. After cooling the balloons, the extract in them was filtered through filter paper.

4-To remove the pigments in the extract, 3.5 ml of 5% zinc sulfate ($ZnSO_4$) and 3.5 ml of normal barium hydroxide ($Ba(OH)_2$) were added to it.

5-The above solution was centrifuged at 3500 rpm for 10 minutes and then the upper part containing the dissolved sugars was poured into a jug flask and its total volume was increased to 100 ml by distilled water.

6-Two ml of the extract was poured into a test tube and one ml of 5% phenol and then 5 ml of pure sulfuric acid were added to each tube.

7-To prepare the control solution, 2 ml of distilled water, 1ml of 5% phenol and 5 ml of pure sulfuric acid were added to another test tube.

8-30 minutes after pouring sulfuric acid in the tubes, due to high heat as a result of the reaction, the adsorption of each solution at 485 nm (by spectrophotometer) was read.

9-Preparation of standard glucose curve: To prepare this curve, concentrations of 50, 100, 200, 250 and 300 mg per liter of glucose were prepared and according to the above method, phenol and sulfuric acid were added to each of the concentrations and then adsorption of each solution in the wavelength was read as 485 nm. The standard curve was plotted by spectrophotometer based on the amount of adsorption versus concentration and the unknown samples (related to different pistachio cultivars) were calculated according to this diagram.

Measuring lipid content of pistachio cultivars

To indicate the probable correlation between lipid content of pistachio cultivars and aflatoxigenic *A. flavus* growth and aflatoxin production, the Soxhlet method was followed in practice. To remove the existent humidity from the kernels, the pistachio kernels were separately desiccated in an oven at 60°C for 48 hours, and then grinded. 3g of each cultivar grinded kernels were separately and exactly weighed, and inside separate filter papers (three repeats were considered for each cultivar). The samples were extracted with 800ml n-hexane (as the solvent for

lipid compounds) added into the distillation apparatus, warmed to the temperature adjusted to the boiling temperature of n-hexane. At the end of lipid extraction from pistachio kernels, the remnants of pistachio nuts of each particular cultivar left on the filter papers were weighed, and based on the primary weights of the samples, the percentage of lipid content of kernels from each cultivar was calculated.

Study on growth rates of A. flavus in pistachio cultivars

Kernel screening assay (KSA)

The method of Cary *et al*. (2011) was used for assessment of pistachio cultivars to *A. flavus* growth. Before this experiment, to ensure non-contamination of pistachio nut samples to *A. flavus*, 30 grams of pistachio kernels are first divided in three 10 gram replicates. The kernels were disinfected with sodium hypochlorite (0.5%) for 1 minute and then rinsed by sterilized water completely. After that, the kernels are immersed in sterile distilled water to absorb the initial moisture for 10 minutes. In the next step, the kernels were placed in sterile Petri plates and added one ml of sterile distilled water. To provide sufficient moisture (saturated), wet pistachio-containing Petri dishes are placed in glass containers with distilled sterile water at the bottom. The container is incubated at 26°C for 8 days and the probability of contamination of pistachios to *A. flavus* was checked. (Moghadam *et al*., 2020).

To calculate the rate of growth and colonization of *A. flavus* in kernels of different pistachio cultivars, 20 gr of kernels of each cultivar (in a completely random design with 3 replications) were surface sterilized by 0.05 sodium hypochlorite and then soaked in distilled water for 10 minutes to absorb the required moisture. Then the kernels were taken into different petri dishes based on their cultivar type and 1ml of the fungal suspension (with a density of 2×10^6 spore/ml) was added to inoculate the kernels. The petri dishes were placed inside plastic containers filled with sufficient distilled water to provide the required moisture. They were then kept at 26°C. Eight days after inoculation, the average percent of growth and colonization of *A. flavus* on kernels of different pistachio cultivars (based on colonized kernel surfaces) were calculated (Moghadam *et al*., 2020; Brown *et al*., 2013). Once the percent of colonization by *A. flavus* was measured, the average colonization percentages of different cultivars were compared and analyzed by SPSS and Duncan's Multiple Range Test.

Extracting and measuring the aflatoxin B_1 produced in contaminated pistachios

Aflatoxin of pistachio samples was extracted by BF method and aflatoxin B1 values were calculated by thin layer chromatography (TLC) and densitometer. Extraction of aflatoxin in the samples was done as follows. 8 days after inoculation of pistachio kernels with *A. flavus*, infected pistachio kernels were dried in an oven at 50°C. 20 g of powdered samples, with 100 ml of methanol: water (55/45 V/V) was shaken for 30 minutes. In the next step, 40 ml of hexane was added and shaken for 15 minutes, then centrifuged at 2000 rpm for 3 minutes. The methanol layer was extracted with chloroform (50ml) thrice. The chloroform phase was filtered through cheesecloth and anhydrous Na_2SO_4 to remove fungal tissue and to dehydrate the extracts respectively. The extracts were evaporated in Bain-marrie. The residue were dissolved in chloroform and analyzed for the presence of aflatoxin on silica gel 60 TLC plates. The developing solvent was chloroform: methanol (97:3). The separated aflatoxin was quantified by fluorodensitometric measurement of extracts spots with Rf value and fluorescence similar to aflatoxin standard. The detection limits of the technique were 2-3 g kg^{-1} of aflatoxins reference standard. Stock solution of aflatoxins were prepared in chloroform and stored in darkness at 4°C (Moghadam *et al*., 2006).

Statistical analysis

Comparison of average amounts of aflatoxin B_1 produced by different pistachio cultivars was done by

SPSS and Duncan's Multiple Range Test. To demonstrate the correlation of nutrient element and protein content of kernels of different pistachio cultivars with *A. flavus* growth and aflatoxin B_1 production, a correlation ratio of (r) was calculated.

Results

Sugar content in kernels of pistachio cultivars

Quantities of Sugar content in kernels of different pistachio cultivars were measured by phenol-sulfuric method and estimated by spectrophotometry. Results being presented in Table 1. As can be seen in this table, there is no significant difference ($P \leq 0.05$) between sugar content of different pistachio cultivars (Table 1).

Table 1. Comparison of average sugar and lipid percentage in kernels of different pistachio cultivars.

Cultivar	Average sugar (%)	Statistical group (α=0.05)	Cultivar	Average lipid (%)	Statistical group (α=0.05)
Akbari	16.06	A	**Kaalkhandan**	58.25	A
Kallehghoochi	15.38	A	**Abbasali**	57.01	AB
Abbasali	15.35	A	**FAS-13-73**	56.12	ABC
Ahmadaghaee	15.2	A	**Kalleh Bozi**	55.82	BCD
Kallehbozi	14.77	A	**Fakhri**	55.78	BCD
FAS-13-73	14.68	A	**Shahpasand**	55.16	BCD
Ovhadi	14.24	A	**Ahmadaghaee**	54.92	BCD
Kaalkhandan	14.05	A	**Kallehghoochi**	54.28	CD
Shahpasand	13.75	A	**Akbari**	53.69	CD
Khanjari	12.65	A	**Ovhadi**	53.39	D

* Similar letters following the averages signify lack of significant difference at 5% level (Duncan's Multiple Range Test)

Lipid content in kernels of pistachio cultivars

The lipid content of kernels of pistachio cultivars was determined by Soxhelt method, and statistically significant differences were found among pistachio cultivars (α = 5%). While Kaalkhandan was of the most abundant lipid contents in their kernels, others like Ovhadi, was of the least lipid contents (Table 1).

Study on A. flavus growth and aflatoxin B_1 production in kernels of pistachio cultivars

The rate of *A. flavus* growth and colonization on kernels of different pistachio cultivars are significantly different at a 0.05 level 8 days after inoculation (Table 2 and Figure 1). Among all tested cultivars, Ahmadaghaee had the lowest and Akbari had the highest rates of resistance against *A. flavus* growth.

Quantities of aflatoxin B_1 produced in pistachio kernels were measured by TLC and densitometer, results being presented in Table 3. As can be seen here, 8 days after inoculation the rates of production of aflatoxin B_1 in different pistachio cultivars were significantly different at a 0.05 level. Among all tested cultivars, Abbasali had the highest and Khanjari had the lowest rates of aflatoxin B_1 production.

Table 2. Comparison of *Aspergillus flavus* growth on kernels of different pistachio cultivars.

Pistachio cultivar	*A. flavus* growth	Statistical group (α = 0.05)
Ahmadaghaee	82.96	A
Ovhadi	76.62	AB
Shahpasand	68.7	BC
Abbasali	65.18	CD
Khanjari	56.97	CDE
FAS-13-73	56.48	CDE
Kaalkhandan	52.40	DEF
Kallehbozi	45.43	EF
Kallehghoochi	42.36	EF
Akbari	41.02	F

* Similar letters following the averages signify lack of significant difference at 5% level (Duncan's Multiple Range Test)

Table 3. Comparison of Aflatoxin production on kernels of different pistachio cultivars.

Pistachio cultivar	Aflatoxin production (µg kg^{-1})	Statistical group (α = 0.05)
Shahpasand	31171	A
Abbasali	30754	A
Ovhadi	29823	AB
Ahmadaghaee	29429	ABC
Akbari	26880	ABCD
Kallehbozi	26410	BCD
Kallehghoochi	26218	BCD
FAS-13-73	25393	CDE
Khanjari	24811	DE
Kaalkhandan	21728	E

* Similar letters following the averages signify lack of significant difference at 5% level (Duncan's Multiple Range Test)

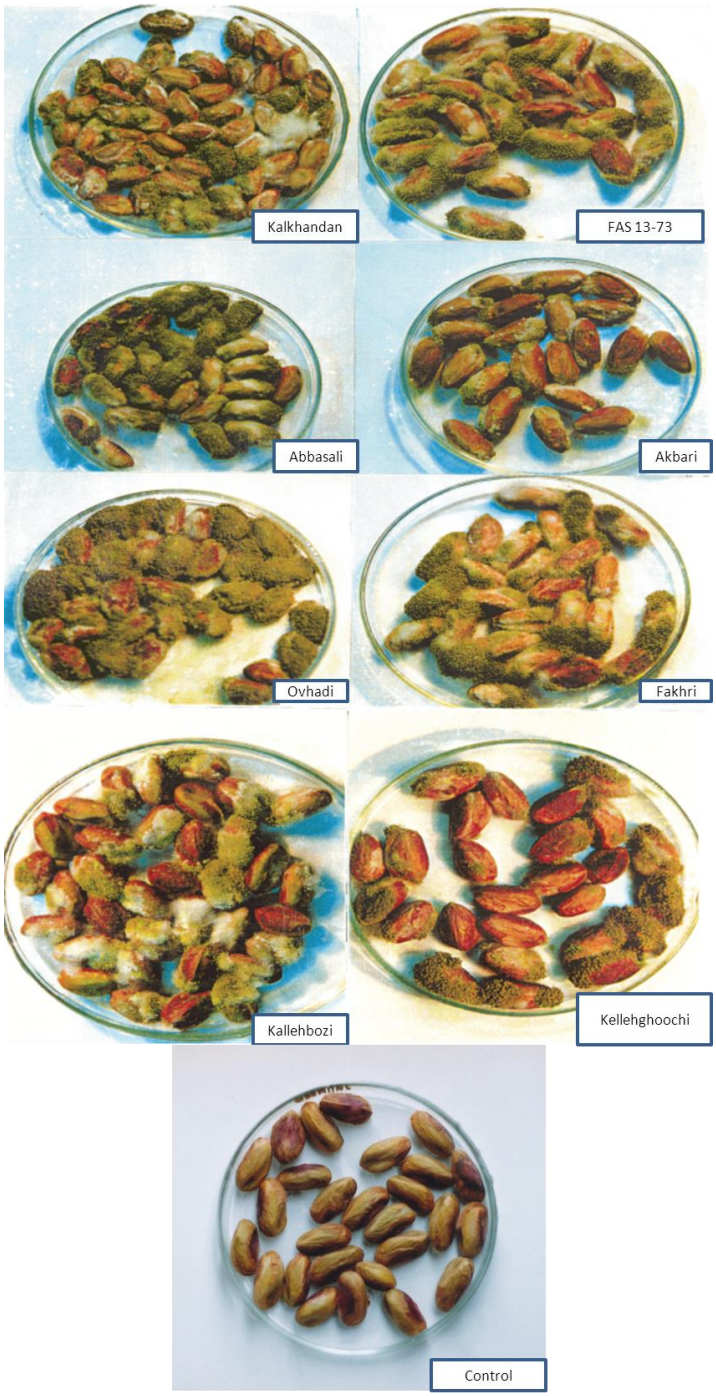

Figure 1. Comparison of *Aspergillus flavus* growth and colonization on kernels of pistachio cultivar (8 days after inoculation).

Study on Correlation between A. flavus Growth and Aflatoxin B_1 Production with Sugar and lipid Content of Kernels of Pistachio Cultivars

To show the possible correlation between Aflatoxin B_1 Production and Sugar and lipid content in kernels of different pistachio cultivars, a correlation ratio (r) was calculated (Table 4). Results indicate that there is no significant correlation between Aflatoxin B_1 Production and sugar contents of kernels of pistachio cultivars. Also, there was no significant correlation between lipid content and aflatoxin production. No correlation was found between *A. flavus* Growth and lipid Content ($r = -0.087^{ns}$; α = 5%). Also, there was not any correlation between *A. flavus* Growth and sugar Content of Kernels of Pistachio Cultivars ($r = 0.23^{ns}$; α = 5%). In other words, increasing or decreasing the sugar and lipid percentage of pistachio cultivars could not be the basis for increasing or decreasing the pistachio resistance to *A. flavus* growth and aflatoxin production. However, there was a significant correlation between *A. flavus* Growth and Aflatoxin B_1 Production in Kernels of Pistachio Cultivars ($r = 0.616^{*}$; α = 5%).

Table 4. Correlation ratio of sugar and lipid content and aflatoxin production in pistachio cultivars

Variable Pairs	a	b	r	r^2
Aflatoxin B_1 production × lipid content	61.563	-0.0002	-0.45^{NS}	0.2027
Aflatoxin B_1 production × sugar content	12.563	0.0000	0.244^{NS}	0.053

Discussion

A. flavus and *A. parasiticus* are the main producers of aflatoxins. Various factors such as genetic characteristics of toxigenic fungi and physicochemical characteristics of its growth medium are involved in aflatoxin production. (Moghadam *et al*., 2020).

Since the discover of aflatoxins, *A. flavus* has been known as the most common fungus producing aflatoxin in food materials, which points to its economic significance (Abbas, 2005). This fungus is capable of influencing a wide range of food materials, continuing to produce aflatoxin in the pre-harvest, post-harvest and storage periods (Elahinia, 2014).

Factors affecting aflatoxin production include the chemical composition of substrate, environmental condition, temperature, moisture, water activity and time. The most important factor here is the chemical composition of food materials. The characteristics of fungi, the chemical composition of the foodstuff, temperature, moisture and time are factors affecting the production of aflatoxin in foods, in which the type of chemical composition of the foodstuff is of particular importance as substrate for *A. flavus* growth and aflatoxin production. In many parts of the world, extensive research has been carried out on various products to determine the role of chemical factors in the growth of fungi, and successful results have been reported. Most research efforts have been focused on peanut, corn and almond (Latha *et al*., 2007; Samapundo *et al*., 2007; Moghadam *et al*., 2020).

Studies have shown that there is a reasonable and significant correlation between the sugar content of peanut kernels and the growth rate of *A. flavus* in different peanut cultivars (Ghewande *et al*., 1993).

Measurement of sugar, protein and phenol content of 38 cultivars of cereals showed that in resistant cultivars there is more protein and phenol content and in sensitive cultivars there is more sugar content (Singh and Behagat, 1990).

Latha *et al*. (2007) showed that out of 21 different peanut genotypes, four genotypes IC-48, J-11, ICGV 89104 and ICGS-76 had the lowest aflatoxin content (<25 ppb) had the highest phenol content (> 1300 µg g^{-1}). Aflatoxin production was negatively correlated

with the phenolic content in peanut kernel (r2 = -0.42) and leaves (r^2= -0.37, p<0.05).

Results of other studies have shown that substrates containing high levels of carbohydrates are quite suitable for aflatoxin production. In general, glucose, galactose, and saccharose were among the carbohydrates best suited for aflatoxin production, while maltose and lactose are less suitable than saccharose. Sorbitol and mannitol have no role in aflatoxin production. Also, saturated fatty acids increase the fungal growth and aflatoxin production processes, while unsaturated fatty acids result in reduced fungal growth and aflatoxin production (Shin and Marth, 1974).

Concerning the nutrient elements and minerals it has been made clear that zinc (Zn) stimulates and increases aflatoxin production. Change of environment Zn from 0 to 10 μg ml^{-1} results in increased aflatoxin production up to a thousand times faster. Moreover, less aflatoxin was produced at 25 μg ml^{-1} densities than the first 10 μg ml^{-1}. Also, manganese (Mn) resulted in increased aflatoxin production, while cupper (Cu) and barium (Ba) totally inhibit the production of aflatoxin (Marsh *et al*., 1975)

Nitrogen stress affects aflatoxin accumulation as well. Payne *et al* (1989) discovered in their studies that aflatoxin accumulation had a negative correlation with corn yield, i.e. the nitrogen in leaves, silk and seeds. Maize inoculated through their silk or body wounds on plants that did not receive any nitrogen contained 28% more aflatoxin than maize plants taking conventional rations of nitrogen fertilizer.

Results of this study confirm that no significant correlation between percentages of sugar and *A. flavus* growth. Also, there was no significant correlation between sugar percentage and aflatoxin production, But given the fact that many other factors including chemical compounds inside pistachio kernels, type and amount of fats (saturated and unsaturated fatty acids), type and amount of carbohydrates, phenolic compounds, and vitamins are capable of interfering in fungal growth and aflatoxin production, any sort of definite conclusion on the correlation of *A. flavus* growth and aflatoxin production with chemical compounds of pistachio kernels requires precise studying of the role of other factors. In other words, more comprehensive and integrated research into other physical and chemical parameters affecting pistachio kernels will enable us to determine the logical correlation(s) between chemical compounds of pistachio kernels and *A. flavus* growth and aflatoxin production.

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