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Relationship between *Aspergillus flavus* growth and aflatoxin B1 and B2 production with phenolic and flavonoid compounds in green hull and kernels of pistachio cultivars

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Abstract

Contamination to *Aspergillus flavus* and aflatoxin is a significant and chronic threat of pistachio production, consumption and exportation in the world. The aims of the present study was evaluation of resistance of pistachio cultivars (Shahpasand, Abbasali,Kale-ghouchi, Khanjari, Akbari and Pesteh-garmeh) and relationship between total phenolic content (TPC) and total flavonoid content (TFC) of green hull and kernels, as antioxidant, to *A. flavus* growth and aflatoxin production. Inoculation of pistachio kernels was done by 2×10^6 spores of toxigenic *A.flavus.* Aflatoxins (B1 and B2), TPC and TFC of the green hull and their kernels were measured by HPLC, Folin-Ciocalteu and aluminum chloride method, respectively. Results showed that Pesteh-garmeh and Akbari cultivars with the lowest and Shahpasand with highest percent colonization of *A. flavus* and aflatoxin production in kernels were respectively resistant and susceptible and other cultivars were intermediate. TPC and TFC were higher significantly in resistant cultivars than susceptible. A negative correlation was observed between TPC, TFC and aflatoxin content in kernel and green hull of pistachio cultivars, as well as TPC, TFC and *A. flavus* growth. These findings show the role of TPC and TFC in resistance of pistachio cultivars with contamination to *A. flavus* growth and aflatoxin production.

Key words: Antioxidants, *Aspergillus flavus*, resistance.

بررسی ارتباط میان رشد *Aspergillus flavus* و تولید آفلاتوکسین های B1 و B2 با ترکیبات فنولی و فلاونوئیدی پوست سبز و مغز ارقام پسته

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چکیده

مسأله آلودگی پسته به قارچ *Aspergillus flavus* و آفلاتوکسین از تهدیدهای مهم در عرصه تولید، صادرات و مصرف پسته در دنیا می‌باشد. در این تحقیق، ضمن تعیین میزان مقاومت ارقام پسته شاهپسند، عباسعلی، کله قوچی، خنجری، اکبری و پسته گرمه به آلودگی ناشی از قارچ *A. flavus* و آفلاتوکسین، ارتباط میان میزان ترکیبات فنولی و فلاونوئیدی پوست سبز و مغز این ارقام، به‌عنوان ترکیبات آنتی اکسیدانی، با میزان رشد قارچ *A. flavus* و تولید آفلاتوکسین مطالعه شد. مایه زنی مغز ارقام پسته با تعداد ۲×۱۰^۶ اسپور در میلی لیتر از یک جدایه توکسین زای *A. flavus* صورت گرفت و میزان آفلاتوکسین، ترکیبات فنولی و فلاونوئیدی مغز و پوست سبز به ترتیب به روش HPLC، فولین سکالتیو و رنگ سنجی با کلرید آلومینیوم تعیین شد. نتایج نشان داد که ارقام پسته گرمه و اکبری دارای کمترین و رقم شاهپسند دارای بیشترین میزان کلنیزاسیون *A. flavus* و غلظت آفلاتوکسین بود و به‌ترتیب به‌عنوان ارقام مقاوم و حساس به آلودگی ناشی از *A. flavus* تعیین شدند. سایر ارقام حد واسط این ارقام قرار گرفتند. میزان ترکیبات فنولی و فلاونوئیدی به‌طور معنی‌داری در ارقام مقاوم بیشتر از ارقام حساس بود. همچنین رابطه منفی معنی‌داری بین میزان ترکیبات فنولی و فلاونوئیدی پوست سبز و مغز پسته ارقام مختلف، با رشد قارچ *A. flavus* و تولید آفلاتوکسین در مغز این ارقام مشاهده شد. این نتایج بیانگر نقش ترکیبات فنولی و فلاونوئیدی در مقاومت ارقام پسته در برابر *A. flavus* و تولید آفلاتوکسین بود.

واژهای کلیدی: آنتی اکسیدان‌ها، مقاومت، *Aspergillus flavus*

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Introduction

Iran has the greatest pistachio genotypic diversity in *Pistacia vera* L. species in the world (Sheibani, 1995). Pistachio production area of Iran is 479368 hectares in 2017 (Ahmadi *et al*., 2017). Kerman province is the main producer of pistachio which produces about 37% of pistachio nuts in Iran. Khorasan-e-Razavi, Yazd, Fars, Semnan and khorasan-e-Jonoobi provinces are also in the next ranks (Ahmadi *et al*., 2017).

Since 1971, the United States has rejected some Iranian and Turkish export pistachio lots for aflatoxin contamination. The theme of aflatoxin and its importance in food industry, especially in the field of dried fruit, have been considered more widely (Elahinia, 2014). In general, aflatoxin production is affected by various factors such as the genetic properties of the fungi and the physicochemical environment in which they grow (Allameh and Razzaghi, 2002). Effective method to reduce toxin producing fungi is using the cultivars and varieties which have less susceptibility to fungal growth and toxin production, (Gradziel *et al*., 2000; Moghaddam *et al*., 2006).

Effective control of aflatoxin infection depends on deep understanding of physiological and environmental factors affecting aflatoxin biosynthesis. It depends on biology and ecology of the fungus and host plant-fungus interaction factors, too (Chen *et al*., 2010). A more fruitful strategy may be to find natural products within the crop that confer resistance to *Aspergillus flavus* growth or prevent aflatoxin biosynthesis, which are natural factors that exist in plant extract and essential oils including phytoalexins and phytoanticipins, TFC and TPC (Abbas, 2005).

Many studies have shown that the resistance of some plant cultivars to the growth of aflatoxin producing fungi is related to the amount of phenolic compounds in the plant, before or after the fungus attack the host (Samapundo *et al*., 2007). To date, most studies have been conducted on the effects of phenolic compounds on the prevention of fungal growth in synthetic culture media and it is necessary to investigate these effects in natural media such as corn and many other products in natural conditions (Samapundo *et al*., 2007).

Phenolic compounds and flavonoids are the most important plant compounds that are considered as natural antioxidants, which due to their hydroxyl group, are capable of eliminate free radicals. (Gulcin *et al*., 2002). They also have anti-mutagenic, anti-cancer and antimicrobial activity (Wojdylo *et al*., 2007) Mechanism of action of phenolic compounds mainly include the elimination of free radicals, metal chelating properties , the ability to regulate gene expression and the role of antoxidative (Neergheen *et al*., 2007). These compounds such as gallic acid, chlorogenic acid, caffeic acid and tannic acid have the potential to inhibit the growth of *A. flavus* and aflatoxin B1 production. (Mahoney *et al*., 2002). San and Chan (1987) investigated the inhibitory effects of phenolic compounds on metabolism of aflatoxin B1 and preventing of its mutagenesis. Different phenolic compounds stopped mutagenesis of AFB1 in *Stamphylium typhimurium*. The results indicate that the inhibitory effect of phenolic compounds on mutagenesis of AFB1 may be due to the inhibition of active enzymes. Mahoney *et al*. (2002) showed that pistachio nut contamination with *A. flavus* and aflatoxin reduced by hydrolysable tannins of pistachio green hulls. This is related to green hulls galatonin. Galatonins are hydrolyzable tannins include glucose esters (or other polyols) and gallic acid. The extract tannin of the green hulls were inhibited the growth of *A. flavus* strongly. In a study, Mahoney and Molyneux (2004) investigated the phytochemical inhibition of walnut compounds in inhibiting *A. flavus* growth and its toxigenicity. The results showed that walnut cultivar Tolar showed high resistance to *A. flavus* growth and aflatoxin production. This inhibition was due to the hydrolysable tannins in the walnut fruit hulls. Samapundo *et al.* showed that the use of natural phenolic compounds (such as vanillic acid and caffeic acid) in maize is effective in preventing the growth of *Fusarium* and *Aspergillus*. Besides, these compounds significantly reduce the production of aflatoxin B1 and fumonisin B1 (Samapundo *et al*., 2007). In a study, Pizzolitto *et al*. (2005) investigated the effects of 10 types of natural phenolic compounds on the growth of *A. parasiticus*. The results showed that isoeugenol, carvacrol and thymol had the greatest effect on inhibition of *A. parasiticus* growth.

Flavonoids are natural phenolic compounds (Penichon *et al*., 2016) occur naturally in fruit, vegetables, grains, tea, and wine. Flavonoids were known for their beneficial effects

on human health. Several potential beneficial properties of flavonoids have since been ascertained, The most important potential clinical effect of this compounds are included, antioxidative effects, direct radical scavenging, anti-inflammatory effects, antitumor effects, antiviral and antifungal effect (Nijveldt *et al*., 2001). Antioxidative effect is the best-described property of flavonoids (Penichon *et al*., 2016). The main aim of the present research is discovering the relationship between TPC, TFC of green hull and kernels, as antioxidant secondary metabolites and *Aspergillus flavus* growth and aflatoxin production.

Materials and Methods

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 *Aspergillus 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 *Aspergillus flavus* was checked. Then, the native isolate of *A. flavus*, which was before isolated from the infected pistachios in Rafsanjan area and its aflatoxigenic potential has been proven (*A. flavus* PRI16499 (Pistachio Research Center), is applied in this research. To calculate the rate of growth and colonization of *A. flavus* on the kernels of different pistachio cultivars, 15 g of kernels of each cultivar (in a completely randomized design with 3 replications) were surface sterilized and then soaked in distilled water for 10 minutes to absorb the required moisture. Pistachio cultivars consisted of Shahpasand, Abbasali, Kale-ghouchi, Khanjari, Akbari and Pesteh-garmeh, the important cultivars in Rafsanjan, Damghan and Mahvelat. Then the kernels were placed 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 for 8 day; the percent of growth and colonization of *A. flavus* on kernels of different pistachio cultivars (based on colonized kernel surfaces) were calculated. Once the percent of colonization by *A. flavus* was measured, the average colonization percentages of different cultivars were compared and analyzed by SPSS software and Duncan's Multiple Range Test (Brown *et al*., 2013; Cary *et al*., 2011).

Aflatoxins measurement of contaminated pistachio nuts

Eight days after inoculation, the contaminated pistachio kernels were dried by oven to prevent further growth of *A. flavus* and aflatoxin production. Assessment of aflatoxin production in pistachio cultivars were measured using Waters e2695 (USA) HPLC, consisting of a chromolith C18, 250 mm × 4.6 mm, column (Phenomenex, USA) equipped with a fluorescence detector (Waters 2475, USA).The mobile phase was water/methanol/ acetonitrile (60:20:20) with a flow rate of 2.5 ml/min. The excitation and emission wavelengths for detection were 365 nm and 435 nm, respectively. The chromatogram of AFB1, AFB2, AFG1 and AFG2 were illustrated in Fig.1. For this purpose, pistachio samples were slurred up with water in a ratio of 1/3 for 15 minutes, and then slurred samples were extracted (30 g) with 90 ml of pure methanol on the blender (Waring, USA) for 3 minutes and filtered through filter paper No. 4. Filtrates (8ml) were mixed with phosphate buffer (42ml). Immunoaffinity columns (VICAM; Milford, MA 01757 USA) were used for purification of samples. Clean up was performed according to the factory instruction. Finally 200 µl of the preparation was injected into the HPLC apparatus(Fani *et al*., 2014). Aflatoxins B1and B2 was measured by comparing the peak areas with a calibration curves obtained by aflatoxin pure standard solutions (Sigma-Aldrich, Milan, Italy). The linearity of the analytical response was checked by analyzing the calibration standards and using seven concentrations over the range 0.4–10 ng/ml aflatoxins B1. In the case of mobile

phase HPLC, the methanol/water (40/60) used for the derivation of potassium bromide, nitric acid and Kobra cell. The chromolite column (10cm) with an internal diameter of 4.6mm (Partisil 5 ODS3, USA) was used. The column temperature was set to 35 ºC with a moving phase of 2.5 mL/min. Fluorescence detector was set at wavelengths ex=365 nm and em=355 nm (Iranian National Standard, No. 5197). Aflatoxin standard chromatogram and AFB1 and AFB2 chromatogram of inoculated pistachio samples by *A. flavus* are showed in Fig. 1.

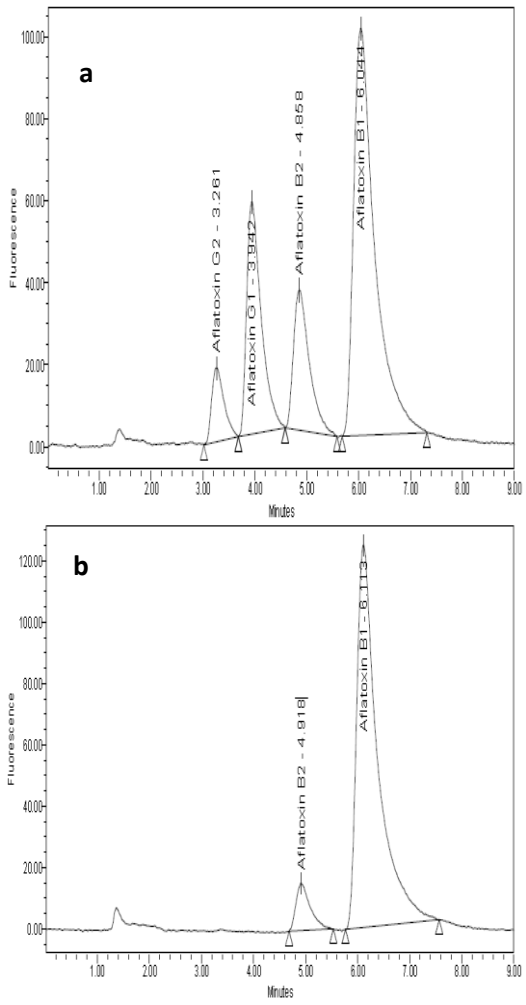

Fig. 1. Aflatoxins standard Chromatogram (a) and AFB1 and AFB2 Chromatogram of inoculated pistachio samples by *Aspergillus flavus* (b).

Determination of total phenolic compound (TPC) of the green hull and kernels of pistachio

The green hull and pistachio kernels extract were obtained using aqueous methanol (1:1) for 72 hours. The content of total phenolic compounds was measured using the Folin-Ciocalteu method. Three test tubes were prepared and 0.5 ml of extract (1000 µg/mL in methanol) was added to each. Then 1.5 ml of distilled water and 0.5 ml of 10% Folin-Ciocalteu reagent were added to each tube. The mixture was stirred vigorously. Then, 1 ml of 5% sodium carbonate was added to each tube and after 120 minutes, the absorbance of the solution was read in 760 nm by a spectrophotometer (Photonix Ar 2015 UV- Vis.). The total phenolic compounds were calculated according to the standard gallic acid (5-20 µg/ml) curve. The result was expressed in mg gallic acid per gram of dried extract.

Determination of total flavonoid compound (TFC) of green hull and kernels of pistachio

The aluminum chloride method was used to measure the total amount of flavonoids. One ml of each extract (100 µg/mL) was mixed with 1 ml of 2% methanolic aluminum chloride solution. After 30 minutes at room temperature and in the dark, the absorbance of all samples was read at 415 nm. Total flavonoids were calculated based on the standard routine (10-160 µg/mL) curve. The results were expressed in terms of routine equivalent per gram of dry extract.

Statistical analysis

All the analyses were performed in triplicate and comparison of average amounts of aflatoxins produced by different pistachio cultivars was done by SPSS and Duncan's Multiple Range Test. To demonstrate the correlation of TPC and TFC of kernels of different pistachio cultivars with *A. flavus* growth and aflatoxin production, a correlation ratio (r) was calculated.

Results

1. *Aspergillus flavus* colonization and aflatoxin production on different cultivars of pistachio kernels and survey on correlation between *A. flavus* growth and aflatoxin production

The isolate of *A. flavus* applied in this research was able to produce AFB1 and AFB2. Colonization of the pistachio kernels of different cultivars resulted significant differences among them (α=5%), for example Shahpasand were heavily colonized (73.6%) by the *A. flavus*, while, on Pesteh-garmeh and Akbari low colonization (50.5 and 47.1 %) were observed (Table 1). Studies through HPLC method indicated significant differences among the aflatoxins contents of the pistachio kernels of different cultivars. Shahpasand had the highest (16082.7 ppb) content of aflatoxins, however, Pesteh-garmeh had the least (9993.7 ppb) but still high amount of aflatoxins (Table 1).

To show the possible correlation between *A. flavus* growth and aflatoxin production in kernels of different pistachio cultivars, a correlation ratio (r) was calculated. Significant positive correlation was found between fungal colonization and growth on pistachio kernels of different cultivars and aflatoxin production , $R^2 = 0.86$; $\alpha = 5\%$, (Fig. 3).

Table 1- Average colonization of kernels (%) of different pistachio cultivars by *Aspergillus flavus* and aflatoxin production.

Cultivar	Average Colonization of kernels (%) by *A. flavus*	Duncan's Group	Rate of Aflatoxins (µg/ kg)	Duncan's Group
Shahpasand	73.6	a	16082.7	a
Abbasali	62.8	b	13964.7	b
Kalleh-ghouchi	60.3	b	12228.3	c
Khanjari	61	b	12360.3	c
Akbari	47.1	c	12037.3	c
Pesteh-garmeh	50.5	c	9993.7	d

In each column the means with the same letter are not significantly different according to Duncan's multiple range test (P<0.05)

2. Total phenolic compounds and total flavonoid compounds of green hull and kernels of pistachio cultivars

TPC amounts of green hull and kernels showed differences in pistachio cultivars. In green hull, Akbari and Pesteh-garmeh cultivars had the highest TPC while Shahpasand showed the lowest TPC. In Kernels, Pesteh-garmeh and Shahpasand had the highest and lowest TPC, respectively (Table 2). TFC concentration had significant difference among the pistachio cultivars (Table 2). Shahpasand and Pesteh-garmeh showed the lowest and highest TFC concentration, respectively. In kernels, Kalle-ghouchi, Abbasali and Shahpasand cultivars had the lowest TFC with no significant difference while Pesteh-garmeh and Akbari showed the lowest TFC concentration.

Table 2- TPC and TFC of green hull and kernels of pistachio cultivars

Cultivar	Average TPC of green hull (GAE/g extract)	Average TPC of kernels (GAE/g extract)	Average TFC of green hull (mg RuE/g extract)	Average TFC of kernels (mg RuE/g extract)
Shahpasand	127.73 d	39.27 e	14.39 e	0.80 bc
Abbasali	151.67 c	43.18 d	13.45 f	0.79 c
Kalleh-ghouchi	157.13 c	45.95 c	22.47 d	0.84 bc
Khanjari	186.65 b	45.56 bc	24.01 c	0.93 b
Akbari	203.81 a	55.79 b	32.25 b	1.14 a
Pesteh-garmeh	205.29 a	66.43 a	41.53 a	1.23 a

In each column the means with the same letter are not significantly different according to Duncan's multiple range test (P<0.05)

3. Correlation between *Aspergillus flavus* growth and TPC and TFC of green hull and kernels of pistachio cultivars

To show the possible correlation between *A. flavus* growth and TPC and TFC of green hull and kernels of pistachio cultivars, correlations were calculated. Results indicate that there is a significant negative correlation between TPC of green hull and *A. flavus* growth on the kernels. ($R^2 = -0.92$; $\alpha = 5\%$). This means that any increase in TPC of green hull of pistachio cultivars tends to reduce fungal growth. Results of statistical reviews also showed that there was significant negative correlation between TPC of kernels of pistachio cultivars and *A. flavus* growth. ($R^2 = -0.85$; $\alpha = 5\%$). In other words, increased TPC of kernels content resulted in reduction of fungal growth (Fig. 2). TFC of green hull showed significant negative correlation with *A. flavus* growth on the kernels. ($R^2 = -0.84$; $\alpha = 5\%$). This means that any increase in TFC of green hull of pistachio cultivars tends to reduce fungal growth. In kernels TFC of pistachio cultivars showed significant negative correlation with *A. flavus* growth ($R^2 = -0.86$; $\alpha = 5\%$). In other words, increased flavonoid contents of kernels content resulted in reduction of fungal growth (Fig. 2).

4. Correlation between aflatoxins production and TPC and TFC of green hull and kernels of pistachio cultivars

To show the possible correlation between aflatoxin production and TPC and TFC content of green hull and kernels of pistachio cultivars, a correlation ratio (r) was calculated. The results of statistical analysis indicated that there were negative significant correlation between TPC and the aflatoxin production in green hull of different pistachio cultivars (R^2 = -0.88; α = 5%), signifying that increased TPC of green hull of pistachio cultivars results in reduced aflatoxin production. Results of statistical reviews also showed that there was significant negative correlation between TPC of kernels of pistachio cultivars and aflatoxins production. (R^2 = -0.87; α = 5%). In other words, increased TPC of kernels content resulted in reduction of fungal growth (Fig. 3).

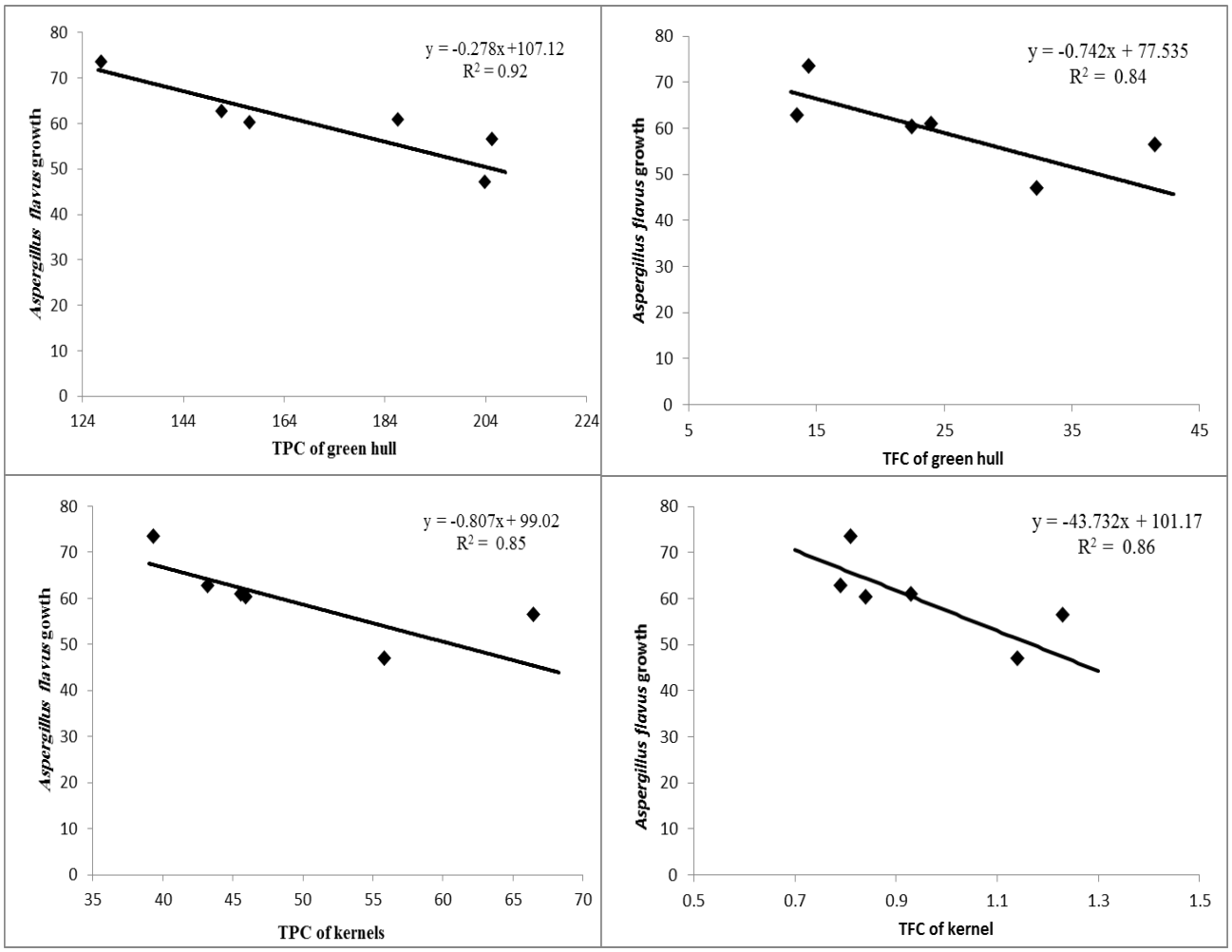

Fig. 2. Correlation between TPC and TFC content of green hull and kernels of pistachio cultivars and *Aspergillus flavus* growth

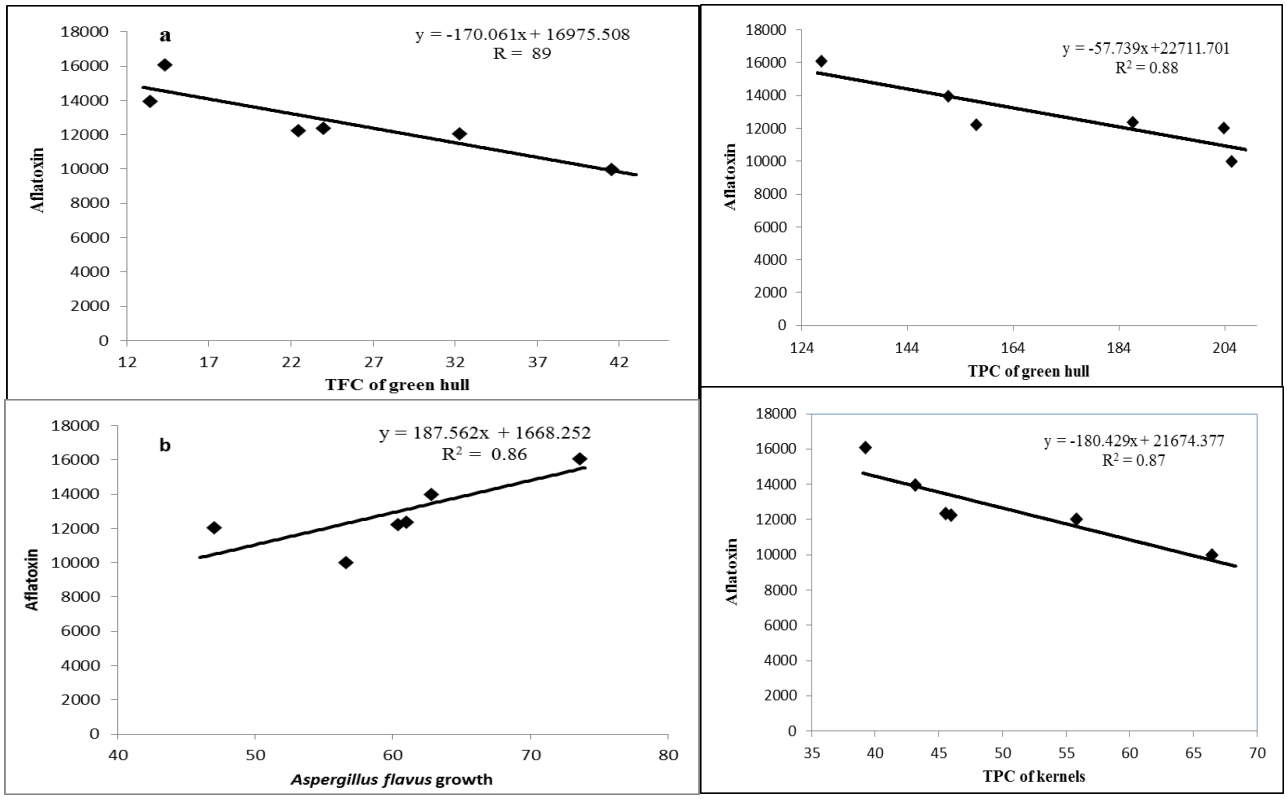

Fig. 3. Correlation between TPC content of green hull and kernels, TFC in green hull of pistachio cultivars and *Aspergillus flavus* Growth with aflatoxins production

The results also indicated that there were negative significant correlations between TFC and the aflatoxin production in green hull of different pistachio cultivars (R^2 = -0.89; α = 5%), signifying that increased TFC of green hull of pistachio cultivars results in reduction of aflatoxin production (Fig. 3). Additionally, Results of statistical reviews also showed some correlation between TFC of kernels of pistachio cultivars and aflatoxins production after colonization by *A. flavus* isolate used in this study.

Discussion

The use of varieties with more resistance to fungal growth and mycotoxin production that are novel pre-harvest strategies that the most promised approaches that have been evaluated (WHO, 2002). The possible role of TPC in inhibiting growth of *Aspergillus flavus* and aflatoxin contamination is one of the most interest subjects in aflatoxin management during recent years (Samapundo *et al*., 2007).

This in part is linked to findings that the resistance of some cultivars of crops such as oilseeds, corn, peanut and tree nuts to *Aspergillus flavus* and *Fusarium* spp and other pathogens contamination has been correlated with their TPC (Siranidou *et al*., 2002; Samapundo *et al*., 2007). In addition, the inhibitory effect of TPC as antioxidant secondary metabolites on growth of some fungi and production of mycotoxins such as tricothecenes, fumonisins, and aflatoxins have been reported several times by many researchers (Backon *et al*., 2003; Beekrum *et al*., 2003).

Phenolic compounds are synthesized in plant cells under favorable environmental conditions, but different plant pathogens, pests and environmental stresses increase the amount of them in the cell (Kliebenstein, 2004). These compounds are valuable physiological evidence in determining the differences between different cultivars (Tattini *et al*., 2006; Arcas *et al*., 2000) reported that high concentrations of phenolic compounds in fruit skin is an appropriate barrier to plant pathogen and pest invasion. Low

levels of aflatoxin in pistachios which have hull compared with peeled pistachio, possibly due to the effects of hull deterrence on *A. flavus* invasion and aflatoxin contamination (Doster and Michailides, 1995). Several reports show the presence of phenolic compounds in the pistachio green hull and high levels of these compounds in the hull compared with the kernel and the antioxidant properties of these compounds (Goli *et al*., 2005 ; Tomaino *et al*., 2010). TPC such as tannins, flavonoids and phenolic acids have been reported to be present in testa of peanut seeds that suggested functioning as performed inhibitors to *A. flavus* growth and aflatoxin contamination. Several researchers attempted to find the correlation between TPC of peanut resistance cultivars against *A. flavus* growth and aflatoxin contamination, then designed developed chemical strategy of screening for *A. flavus* growth and aflatoxin contamination resistance in agricultural crops such as peanut (Liang *et al*., 2006).

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 food stuff 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). Premlata *et al*. (1990) investigated on the role of protein and phenol contents of 38 cultivars of legumes in resistance of the cultivars to *A. flavus* growth and aflatoxin production, the results of this study showed that the protein and phenol content in resistant cultivars were more than in sensitive cultivars. (Premlata *et al*., 1990). In a study carried out by Latha *et al*. (2007) on 21 genotypes of peanuts, it was found that IC-48, J-11, ICGV 89104 and ICGS-76 genotypes had the lowest levels of aflatoxin (< 25 ppb) and the highest amount of phenols. The aflatoxin production had a negative correlation with phenol contents of the kernels and leaves. The potential of phenolic compounds to inhibit the growth of several plant pathogenic fungi has already been proven. Siranidou *et al*. (2002) showed that wheat cultivars with high resistance to fungal growth contain plenty of phenolic compounds. Accumulation of phenylpropanoids after contamination with various fungi has been considered as an important factor in resistance to pathogens in cereals. Beekrum *et al*. (2003) proved the inhibitory effect of various phenolic compounds, such as vanillic acid and caffeic acid, in preventing the growth of *Fusarium verticillioides*. Studies by Samapundo *et al*. (2007) Showed that the use of natural phenolic compounds (such as vanillic acid and caffeic acid) in maize is effective in preventing growth of *Fusarium* and *Aspergillus* fungi. The use of these compounds also significantly reduces the production of aflatoxin B1 and fumonisin. Pizzolitto *et al*. (2015) examined the effect of 10 types of natural phenolic compounds on the growth of *A. parasiticus*. According to the results, isoeugenol, carvacrol and thymol compounds had the most effect on preventing growth of *A. parasiticus* and compounds such as creosol, p-cresol, o-cresol and vanillin did not show any effect on the growth of these fungi. Results of this study confirm in laboratory conditions (artificial inoculation of different pistachio cultivars with *A. flavus*), there was significant positive correlation between the growth rate of fungus and the production of aflatoxin that was contrary to the results of other research on corn and peanut in normal and field conditions. In the laboratory conditions of the present study (artificial inoculation of the pistachio nuts), due to control of environmental conditions including moisture and temperature and other parameters, there is a direct and significant correlation between the growth rate of fungi and the production of aflatoxin. Also results demonstrated a significant negative correlation between *A. flavus* growth and total phenol contents of green hull and kernels of pistachio cultivars. Additionally, there was a significant negative correlation between total flavonoid contents of green hull and kernels of pistachio cultivars and *A. flavus* growth. Also, the results of this research revealed that there was a significant correlation between aflatoxin production in kernels of different pistachio cultivars and total phenol contents of green hull and kernels of pistachio cultivars. Additionally, there was a significant negative correlation between total flavonoid contents of green hull of pistachio cultivars and aflatoxin production. There was not observed significance

correlation between aflatoxin production and total flavonoid contents of kernels of pistachio cultivars. The result of the present research demonstrated that TPC and TFC as antioxidant secondary metabolites in green hull and kernels of pistachio cultivars play a key role in acquiring more resistance for *A. flavus* invasion and aflatoxin contamination in pistachio cultivars (Latha *et al*., 2007). But given the fact that many other chemical composition of pistachio kernels, such as protein, carbohydrate, lipids contents and amount of fatty acids (saturated and unsaturated fatty acids), amino acids and type and amount of carbohydrates, and vitamins, the physicochemical of testa as a barrier again *A. flavus* invasion 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. In this study it is concluded that total phenol content (TPC) and total flavonoid content (TFC) of green hull and pistachio kernels, significantly affect the fungal growth and aflatoxin production. Further studies are suggested to reach mathematical models to predict the antifungal activity of the phenolic compounds from their molecular properties, or study the resistance mechanisms by analyzing gene expression of pistachio genes.

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