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**ORIGINAL ARTICLE**

# Population Density of *Aspergillus* Section *Flavi* and Aflatoxin Content in Different Types of Pistachio Nuts

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**KEYWORDS**

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**ABSTRACT:** Infection of pistachio nuts with *Aspergillus flavus* and aflatoxin is one of the serious and important threats in the process of production, export, and consumption of pistachios in the world. Aflatoxins are secondary metabolites that are mainly produced by *A. flavus* and *A. parasiticus*. Aflatoxins are generally produced before harvesting and under horticultural conditions. Early-splitting pistachios are one of the most significant sources of contamination to aflatoxigenic fungi in the orchard. In the present study, the population density of *Aspergillus* section *Flavi* and aflatoxin content were investigated in early-splitting, irregular cracking, and intact pistachios of the Shahpasand cultivar. Sampling was done from the pistachio orchard of Damghan Pistachio Research Station in the first half of August, and pistachio kernel contamination and population density of the fungus were investigated. Pistachio samples were cultured on an AFPA medium using a serial dilution method (completely randomized design with three repetitions). *Aspergillus* section *Flavi* colonies were identified and counted and after three days at 28°C and dark. Aflatoxin contents of pistachio kernels were quantified by high-performance liquid chromatography (HPLC) method. The results were statistically analyzed by SPSS statistical software and the means were compared using Duncan's multiple range test. The results showed that the highest and the lowest contamination rates for *Aspergillus* section *Flavi* and aflatoxin have belonged to early-splitting and intact nuts, respectively ($P \leq 0.05$). The contamination rate in early splitting pistachios is much higher than in irregular cracking and intact kernel pistachios.

## INTRODUCTION

Currently, more than 150 pistachio cultivars in Iran are identified morphologically and stored in pistachio collections. However, the number of cultivars and genotypes that have not yet been identified, but are present

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in pistachio orchards in the country, reaches more than 150 cultivars. In other words, more than 300 pistachio cultivars are currently cultivated in different parts of the country, which can be used as a unique genetic resource in breeding programs [1]. Abbasali, Khanjari, and Shahpasand from the Damghan area are important cultivars in the region that have prominent characteristics. Shahpasand cultivar is related to Damghan, which has a semi-extensive growth habit and weak growth strength. This cultivar is early flowering and precocious, and has the fruit a drawn-oval, pink-dark red color, weighs about 1.14 grams and the ratio of the kernel to shell is 51.75% [2]. The low percentage of fruit porosity and the high percentage of splitting fruit are the advantages of this cultivar and the high cracking of the green hull and the non-uniform ripening of the fruit are its disadvantages. The most important factor that causes fungi to penetrate the pistachio fruit is the cracking of the green hull on the pistachio kernel in the orchard and early splitting is one of the most important types of cracks. Early-splitting, which is one of the most important causes of pistachio contamination with *Aspergillus flavus* as the main aflatoxin producer, occurs in fruits whose upper crack along the splitting groove of the shell prematurely, this exposes the pistachio kernel to mold and insect pests [3, 4]. Due to the onset of early-splitting occurs before physiological maturity and more than one month before harvest in the orchard, there is ample opportunity for fungi to grow and produce aflatoxins. Fifteen to 45% of early-splitting pistachios are formed more than 4 weeks before harvest, and 10 to 30% of them 2 weeks before harvest [5]. The existence of *A. flavus* has been proven in growing pistachio fruits before harvest [6, 3]. The infection has been reported to be associated with the exposure of pistachio kernels to airborne spores due to cracking of the green hull [7]. It has also been found that the infection with *A. flavus*, *A. parasiticus*, and aflatoxin in early-splitting pistachios infected with navel orange worm (NOW) (*Amyelois transitella*) pest is higher than in early-splitting pistachios uninfected with this pest. In addition, pistachios with the intact hull (without cracking) and not splitting pistachios are aflatoxin-free [8]. Investigation of contamination status of early-splitting and irregularly cracked pistachios showed that early-splitting pistachios with wrinkled superficial hull compared to early-splitting pistachios with soft superficial hull were infected more than twice to *A. niger*, and more than three times with *A. parasiticus* and *A. flavus*. Pistachios infected with *A. transitella* also accounted for 84% of the total aflatoxin. The hull of early-splitting pistachios often contains low amounts of aflatoxins [9]. There is a direct relationship between early-splitting pistachios and aflatoxin contamination. The amount of aflatoxin in the kernels of early-splitting pistachios with the wrinkled and dry hulls is more than in early-splitting pistachios with the smooth and soft hulls. The amount of aflatoxin in the early-splitting and irregularly cracked pistachios with the wrinkled and dry superficial hull is much higher than in cracked pistachios with smooth and soft hull [10]. Studies on pistachio infestation in the orchard have shown that the main causes of pistachio contamination with aflatoxin are early-splitting and green hull rupture, so that in 22% of samples of such pistachios that were free of pests, aflatoxin was observed, and pest infestation has increased aflatoxin levels by up to 75% [8]. Numerous studies showed that early-splitting and late harvest is two important factors in the contamination of pistachios with aflatoxin in the orchard. According to the researchers, an early-splitting nut is 50 times more likely to be infected with aflatoxin than normal nuts [4, 9]. A study conducted on contamination of early-splitting found that a decrease in soft-hull moisture indicates a longer time to crack and a higher risk of infection, so this is 3 times more likely in early-splitting kernels with dry and wrinkled hull than in fresh hull kernels. In addition, the amount of toxins in nuts with the dry and wrinkled hulls is much higher [11]. It has been shown the presence of *Aspergillus* species propagule in male inflorescences of pistachio trees, cracked fruits, plant debris, and animal manures [12, 13]. It has been concluded that different rootstocks affect on the percentage of early-splitting so that *Pistacia atlantica* rootstocks have the highest percentage of early-splitting and domestic rootstocks have the lowest percentage of early-splitting. Different rootstocks also affect the

percentage of irregularly-cracked pistachios, so that *P. atlantica* rootstocks have the highest amount and *P. vera* rootstock has the lowest amount of irregularly-cracked pistachios. Numerous studies have shown that infection of different terminals with *A. flavus* and *A. niger* fungi can be affected by various factors such as the type of pistachio processing terminal (traditional or mechanized) and the type of pistachio entering the terminal. Important factors influencing this issue are the abundance of cracked pistachios (especially early-splitting and irregularly cracked pistachios with dry and wrinkled hull) and pistachios piled on the ground in a pistachio mass entering a terminal [14, 15].

## MATERIALS AND METHODS

### *Evaluation of population density of Aspergillus section Flavi in pistachios*

To investigate the population density of *Aspergillus* section *Flavi* in early-splitting, irregular cracking, and intact pistachios of Shahpasand cultivar, sampling was performed from the pistachio orchard of Damghan Pistachio Research Station (Figure 1). This orchard had 20-year-old trees of commercial cultivar Shahpasand and proper management of the principles of horticulture, irrigation, nutrition, and control of plant pests and diseases was applied in this orchard. Ten trees in different directions were randomly selected and labeled. In early August 2020-2021, the beginning of the formation of early-splitting nuts on pistachio trees was examined weekly. Since the Shahpasand cultivar is one of the earliest-ripening pistachio cultivars in the country, the crop was harvested in the first half of August according to the custom of the region. At harvest time, the whole crop of each tree was harvested carefully and without damaging the hull of the fruit. All harvested pistachios were inspected and early-splitting, irregular cracking and intact hulls of each tree were isolated, counted, and recorded. Pistachio nuts were stored in the refrigerator until cultured on the AFPA medium.

Because the distribution of contamination in different parts of the samples is distinct from each other, and estimation of *Aspergillus* section *Flavi* contamination requires a homogeneous and completely uniform sample, pistachio samples were first ground and then cultured in AFPA specific culture medium using serial dilution method. Aspergillus Flavus and Parasiticus Agar (AFPA) differential culture medium is used to screen and identify species belonging to the *A.* section *Flavi* [16, 17]. In this culture medium, two species *A. flavus* and *A. parasiticus* can be identified from each other by producing distinct spores in yellow to olive-green and light orange behind of petri dish [18]. AFPA culture medium is used to detect species belonging to the *Aspergillus* section *Flavi*. The medium relies upon the formation of a bright orange-yellow reverse pigment by *Aspergillus flavus* and related species. Because they can be easily distinguished from other species by creating a light orange color behind the petri dish [19]. (Figures 2 and 3).

For this purpose, 10 g of ground pistachio kernels from each sample was added to 90 ml of 0.1% peptone water and shaken for 20-40 minutes. One-tenth of a milliliter of $10^{-1}$ and $10^{-2}$ dilutions was spread on the surface of the Petri dishes containing AFPA medium (completely randomized design with three replications). The Petri dishes were incubated at 28°C for three days. The *A. flavus* colonies were isolated, identified, and counted. By counting the number of *A. flavus* colonies on the surface of Petri dishes, contamination rates in different samples (early splitting, irregular cracking, and intact pistachios) of pistachios were compared. AFPA culture medium has the property of preventing sporulation of colonies of species belonging to the section *Flavi*, so that fungal colonies are distinct and easily distinguishable from each other and can be counted. The obtained data were statistically analyzed by SPSS statistical software version 22 and the means were compared by Duncan's multiple range tests at a 5% level.

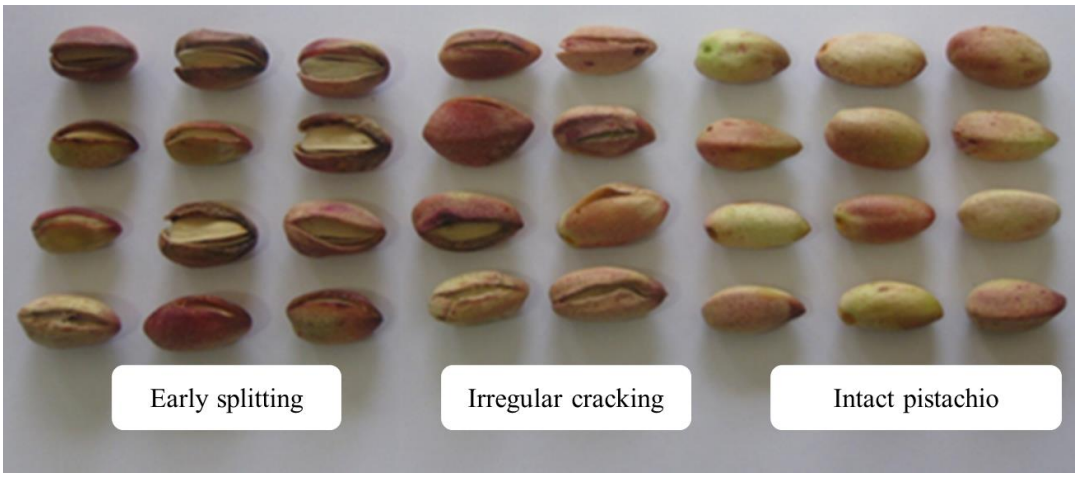


**Figure 1.** Comparison of early-splitting, irregular cracking, and intact pistachios

**Figure 2**. Growth of *Aspergillus* section *flavus* on AFPA medium

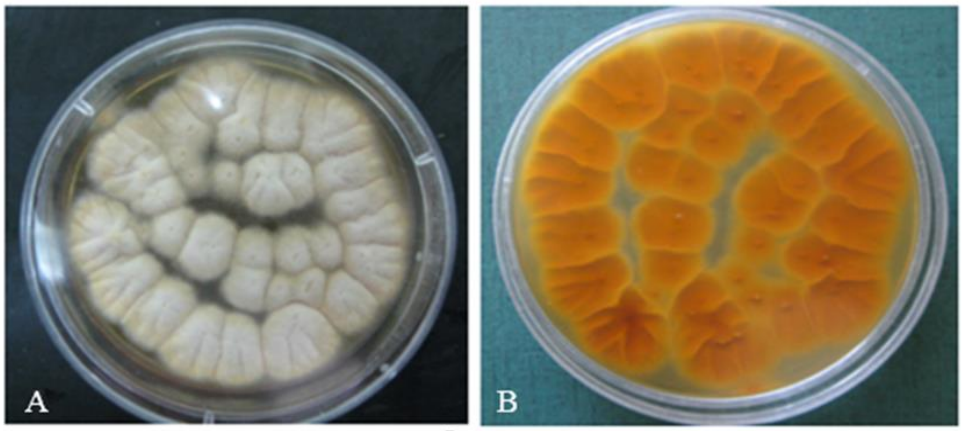


**Figure 3.** Growth of *Aspergillus flavus* on and behind the Petri plate of AFPA medium

***Quantification of aflatoxins produced in pistachio kernels***

For quantification of the aflatoxin, pistachio kernels were dried and the aflatoxin content of samples was measured by using Waters e2695 (USA) HPLC, consisting of a chromolith C18, 100 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 $min^{-1}$. The excitation and emission wavelengths for detection were 365 nm and 435 nm, respectively. The limit of detection (LOD) for AFs was 0.3 mg $ml^{-1}$. The chromatogram of $AFB_1$, $AFB_2$,

$AFG_1$, and $AFG_2$ are shown in Figure1. For this purpose, pistachio samples were slurred up with water in a ratio of 1/3 for 15 minutes, then slurred samples were extracted (30 g) with 90 ml of pure methanol on a Waring blender (Waring, USA) for 3 minutes and filtered through Whatman paper No. 4. (8 ml) were mixed with phosphate buffer (42 ml). Immunoaffinity columns were used for the purification of samples. First, 20ml of phosphate buffer was passed (transmitted) through the column to read it, then 25 ml of the extract mixed with the phosphate buffer was passed (transmitted) through the column, and, the column was again washed with 20 ml of phosphate buffer. After drying the column, 1500 µl of methanol (with the purity special for liquid chromatography) was passed through the column. By one minute, 750µl of methanol was again passed through the column. After collecting the total methanol phase, 1750 µl of water was added to it, and finally, 200 µl of the preparation was injected into the HPLC apparatus. Aflatoxins $B_1$, $B_2$, $G_1$, and $G_2$ were measured by comparing the peak areas with 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 of 0.4–2.7 ng $ml^{-1}$ aflatoxins $B_1$. In the case of mobile phase HPLC, the methanol/water (40/60) was used for the derivation of potassium bromide, nitric acid, and Kobra cell. The chromolite column (10cm) with an internal diameter of 4.6 mm (Partisil 5 ODS3, USA) was used. The column temperature was set to 35ºC with a moving phase of 2.5 ml $min^{-1}$. The fluorescence detector was set at wavelengths ex=365 nm and em=355 nm [20].

## RESULTS

### *Comparison of population density of Aspergillus section Flavi in different pistachios*

The results showed that the rate of pistachio kernel contamination with *Aspergillus* section *Flavi* in early-splitting nuts, irregularly cracking and healthy nuts were 8140, 3280, and 640 CFU/g, respectively. These results indicate that the highest and lowest fungal densities were observed in the samples of early-splitting and samples with intact hulls, respectively. (Table 1). As can be seen in the table, the rate of contamination in early splitting pistachios is much higher than in irregularly cracking and intact pistachios.

**Table 1.** Comparison of mean difference in colony number of *Aspergillus* section *Flavi* in kernels of early-splitting, irregular cracking, and intact pistachios.

| Pistachio type | Mean of colony number of *Aspergillus* section *Flavi* (CFU/g) | Grouping variables (α =5%) |
|---|---|---|
| **Early-splitting nuts** | 8140 | a |
| **Irregular cracked nuts** | 3280 | b |
| **Nuts with intact hulls** | 640 | c |

Average followed by the same letter is not significantly different at a level of 5%, by Duncan's Multiple Range Test.

### *Aflatoxins production in early splitting, irregular cracking, and intact pistachios*

Assaying aflatoxin production level in kernels of early-splitting, irregular cracking, and intact pistachio is shown in Table 2. The results confirmed the fungus density test. The results of comparing the mean of the data showed a significant difference between the treatments (early-splitting, irregular cracking, and intact hulls samples) at the level of 5%. According to the results, the rate of aflatoxin contamination of pistachio kernels in early splitting pistachios, irregular cracking, and intact hulls samples were 66, 33, and 0%, respectively, which shows that the highest amount was observed in early-splitting samples and then

samples with irregular cracks. Samples with intact hulls showed no contamination.

**Table 2.** Frequency of contamination samples to aflatoxin in the kernel of early-splitting, irregular cracking, and intact pistachios.

| Pistachio type | Contamination samples frequency (%) | Grouping variables (α=5%) |
|---|---|---|
| **Early-splitting nuts** | 66% | c |
| **Irregular cracked nuts** | 33% | b |
| **Nuts with intact hulls** | 0% | a |

Average followed by the same letter is not significantly different at the level of 5%, by Duncan's Multiple Range Test.

## DISCUSSION

Aflatoxins are fungal secondary metabolites that are produced in many crops, mainly corn, oilseed, and nuts in the pre-harvest stages; however, delay in harvest, lack of proper processing, the presence of contaminated fruits, and unfavorable storage conditions can also intensify the contamination in the post-harvest stages [3, 21, 22, and 23]. Therefore, to prevent aflatoxin and *Aspergillus* contamination, on-farm crop management and ensuring optimal post-harvest storage conditions are of particular importance [24].

Under certain temperature and humidity conditions, pistachio fruits can be contaminated by aflatoxin-producing isolates belonging to *Aspergillus* section *Flavi*, resulting in biosynthesis and accumulation of aflatoxin in pistachio fruit [25]. Of the approximately 18 known aflatoxins, aflatoxins $B_1$, $B_2$, $G_1$, and $G_2$ have been reported in agricultural products [26] to have unfavorable effects on human and domestic animal health [22, 27]. Aflatoxin $B_1$ is the most toxic and abundant type, which according to the classification of the International Agency for Research on Cancer, is considered a group 1 carcinogen and suppressor of the immune system in humans [25, 28].

The time interval between flowering and fruit set, when the shell of the fruit is not yet fully formed, as well as the early splitting and cracking of the hull during the ripening time of the fruits are known as growth stages susceptible to *A. flavus* contamination and aflatoxin production. By cracking in the hull, the fruit kernel is exposed to contamination by microorganisms such as *A. flavus*, and because at this stage the moisture content of the pistachio kernel varies from about 30 to 40% and is favorable for the growth of fungi, it is possible to grow *A. flavus* and produce spores. Early-splitting pistachios with dry and semi-dry hulls are more infected with *Aspergillus* than soft-hulled early-splitting pistachios [29]. Of course, early-splitting and hull cracking are also related to the type of rootstock, cultivar, and irrigation management, and any delay in pistachio harvest can lead to increased aflatoxin contamination [30, 31]. Insect damage which causes physical damage and the transfer of fungal spores to the fruit can also lead to aflatoxin contamination [4, 9, 24, and 32].

Aflatoxin contamination in pistachios has caused major problems in the United States, Asia and Africa. For example, in September 1997, the European Union returned a large consignment of Iranian pistachios due to high aflatoxin contamination [33]. The researchers' findings suggest that control of aflatoxin contamination is likely to be possible through a combination of biological control methods, field farming, and the application of resistant cultivars in crops such as maize and resistant transgenic cotton [34, 35]. The results of the present research showed that the highest amount of aflatoxin contamination was observed in early-splitting samples. Pistachios with irregular cracks were in the next degree. Any contamination was observed in pistachios with intact hulls. These results are consistent with previous research conducted by various researchers, which is summarized as follows. Research shows that pistachios suspected of being

aflatoxin contaminated in the orchard include the following groups: 1- early-splitting pistachios with wrinkled and dry hull; 2- irregularly cracked pistachios with wrinkled and dry hull; 3- early-splitting pistachios with soft and smooth hull; 4- irregularly cracked pistachios with soft and smooth hull; 5- pistachios affected by pests; 6- pistachios with green hull damage, including pistachios in contact with the ground, pistachios with physiological problems or other factors [10].

Early-splitting, which is one of the most important causes of pistachio contamination with *A. flavus*, occurs in fruits whose upper crack along the splitting groove of the shell prematurely; this exposes the pistachio kernel to mold and insect pests [3, 4].

Due to the onset of the early-splitting disorder in pistachios before physiological maturity and more than one month before harvest in the orchard, there is ample opportunity for fungi to grow and produce aflatoxins. Fifteen to 45% of early-splitting pistachios are formed more than 4 weeks before harvest, and 10 to 30% of them 2 weeks before harvest [4]. The existence of *A. flavus* has been proven in growing pistachio fruits before harvest [3]. The infection has been reported to be associated with the exposure of pistachio kernels to airborne spores due to cracking of the green hull [7]. It has also been found that the infection with *A. flavus*, *A. parasiticus*, and aflatoxin in early-splitting pistachios infected with pests is higher than in early-splitting pistachios uninfected with this pest [8].

Investigation of the contamination status of early-splitting and irregularly cracked pistachios showed that early-splitting pistachios with wrinkled superficial hull compared to early-splitting pistachios with soft superficial hulls were infected more than three times with *A. parasiticus* and *A. flavus*. The green hull of early-splitting pistachios often contains low amounts of aflatoxins [9]. Numerous studies conducted by Doster et al. show that early-splitting and late harvest are two important factors in the contamination of pistachios with aflatoxin in the orchard. According to the researchers, an early splitting seed is 50 times more likely to be infected with aflatoxin than healthy seeds [9].

The results of the present study showed that the amount of fungus and aflatoxin decreased in early-splitting, irregular cracking, and intact hulls samples, respectively. This indicates that the samples with intact hulls showed the least contamination so the lowest fungal density was observed in these samples and no aflatoxin was observed in them. Considering that early-splitting and late harvest are two important factors in the contamination of pistachios with aflatoxin in the orchard, therefore, first of all, in selecting a suitable cultivar for cultivation in different pistachio growing regions of the country, early-splitting of the cultivar should be considered as an important factor. Secondly, in cultivars such as Shahpasand, which is the earliest-ripening pistachio cultivars and the percentage of early-splitting is very high, it is recommended to harvest as soon as the crop ripens, to reduce the risk of fungal infection and subsequent production of aflatoxins.

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### *Conflict of interests*

All the authors declare that there is no conflict of interest in the study.

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