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Evaluation of Contamination of *Aspergillus flavus* and Aflatoxin Production in Pistachio Cultivars and Investigation of a Chemical Controlling Method

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Abstract

In order to study the contamination of *Aspergillus flavus* and aflatoxin production in pistachio cultivars in the Semnan province, eight cultivars of pistachio were collected from major pistachio growing areas. Using the serial dilution method, ground pistachio kernels were inoculated on plates containing AFPA medium and incubated at 28° C. This experiment was performed using three replications in a completely randomized design. After three to seven days, the number of *A. flavus* colonies were identified and enumerated. Also, aflatoxin B_1, B_2, G_1 and G_2 contents of the samples were analyzed by HPLC method. On the other hand, the effect of two chemical fungicides as a control method on the growth of *Aspergillus flavus* and aflatoxin production in pre-harvest pistachio cultivars was assessed under *in vivo* conditions. For this purpose, an orchard that was under cultivation by the most contaminated cultivar was selected, and a completely randomized design was carried out in the field. Two fungicides (tebuconazole 25% and mancozeb 80%) were applied at an application rate of 1 and 2 L or Kg ha^{-1}, respectively. Aflatoxin B_1 and B_2 contents of the samples were analyzed using the HPLC method. The obtained results showed that there was a significant difference in *A. flavus* colonies number in different pistachio cultivars. Among these cultivars, Owhadi had the highest amount of contamination, and Akbari had the lowest contamination. The results showed that the contents of aflatoxin B_1 and B_2 were observed in Owhadi cultivar. , Tebuconazole 25% and mancozeb 80% reduced *A. flavus* growth compared to the control. However, this reduction was not significant. The obtained results of aflatoxin analysis showed that these two fungicides reduced the amount of aflatoxins B_1 and B_2 in pistachio cultivar, though there was not a significant reduction.It was concluded that the use of chemical fungicides were ineffective in preventing *A. flavus* growth and aflatoxin production in pistachio cultivars under *in vivo* conditions.

Keywords: Aflatoxin, *Aspergillus flavus*, Fungicides, HPLC, Pistachio, Semnan province.

Introduction

Aspergillus flavus is a saprotrophic and pathogenic fungus colonizing tree nuts, cereal grains and legumes. Post-harvest infection usually occurs during harvest, storage, and transit. *A. flavus* infections can develop while hosts are still in the field (pre-harvest) but often demonstrate no symptoms (dormancy) until post-harvest storage or transport. *Aspergillus flavus* is one of the most prevalent storage fungus colonizing pistachio kernels. Many strains produce significant amounts of toxic compounds known as aflatoxins, which, when consumed, are toxic for animals and humans (Onial *et al*., 2015). Aflatoxins are secondary metabolites produced by some species of Aspergillus, particularly *A. flavus* and *A. parasiticus* (Jalali *et al*., 2012). Aflatoxins

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B_1, B_2, G_1 and G_2 are the most toxic and carcinogenic recognized compounds among mycotoxins that contaminate agricultural products. Among these four major aflatoxins, B_1 possesses the highest toxic level and then G_1, B_2 and G_2 aflatoxins show lower toxicities, respectively (Rahimi *et al*., 2007). Consumption of food contaminated with aflatoxin may generate irreparable effects in animals or humans (Yabe *et al*., 1993). On the other hand, pistachio is a very important economic aspect of agricultural production in Iran and brings the highest currency income among non-oil exporting products. Since the main problem during export is contamination with aflatoxin, controlling *A. flavus* growth and aflatoxin production in pistachios is critically important. Khodavaisy *et al*. (2012) evaluated the existence of *A. flavus* in pistachios and peanuts in Sanandaj, Iran. Their results showed that fungi were detected in almost 72% of the samples. *A. flavus* fungus was the most predominant isolate from pistachio (22%) and peanut (19%) samples. Aminshahidi (1997) studied aflatoxigenic *Aspergillus* species in native contaminated pistachios of Iran and investigated their capacity in producing aflatoxin. The results showed that most of the examined samples were contaminated with *A. flavus* and *A. parasiticus* molds and aflatoxin. Brenneman *et al*. (1993) investigated effects of diniconazole on *Aspergillus* populations and aflatoxin formation in peanuts. Treatment with diniconazole had no effect on populations of the *A. flavus* group in the soil or shells and reduced populations in seed. Aflatoxin concentrations were significantly correlated to *A. flavus*-group populations in both shells and seed. Santos *et al*. (2011) showed an *in vitro* effect of some fungicides on growth and aflatoxins production by *Aspergillus flavus* isolated from Capsicum powder. It was concluded that the most efficient fungicide in reducing growth is not always the best choice for preharvest treatments, because it may promote aflatoxin production. Thus, the best fungicide is the one that can simultaneously prevent growth and aflatoxin production. Wheeler *et al*. (1991) investigated effects of chlobenthiazone on aflatoxin biosynthesis in *Aspergillus flavus*. Chlobenthiazone had a strong inhibitory effect on the synthesis of aflatoxin B_1 by strains of fungus. *A. flavus* failed to produce aflatoxins at chlobenthiazone concentrations above 8 microgram/ml. Krishnamurthy and Shashikala (2006) evaluated the inhibition of aflatoxin B_1 production of *Aspergillus flavus*, isolated from soybean seeds by fungicide captan and certain natural plant products. Their results showed that all the treatments were effective in controlling aflatoxin B_1 production. Captan reduced the level of aflatoxin B_1. All the natural product treatments applied were significantly effective in inhibiting aflatoxin B_1 production on soybean seeds by *A. flavus*. They suggested that these natural plant products may successfully replace chemical fungicides and provide an alternative method to protect soybean and other agricultural commodities from aflatoxin B_1 production by *A. flavus*. Formenti *et al*. (2012) researched the condition of growth and aflatoxins production by *Aspergillus flavus* using anti-fungal compounds. The temporal efficacy of three different chemical fungicides in reducing growth and toxin production by isolates of *A. flavus* was studied. Their results showed that all the fungicides significantly inhibited mycelial growth compared to the control. An inhibitory effect of all fungicides generally improved with increasing concentration. Also, all the fungicide treatments resulted in a significant reduction in aflatoxin B_1 production when compared to the control. Considering the economic importance of pistachio in Iran, the aim of our study was to evaluate pistachio cultivars contamination in relation to *A. flavus* growth and aflatoxin production in the Semnan province. This study also aimed to evaluate the efficacy of some fungicides for controlling *A. flavus* growth and aflatoxin production.

Materials and Methods

The first step in this study was to select a suitable sample. Eight cultivars of pistachio from different pistachio growing areas of the Semnan province, including Owhadi, Ahmadaghaii, Shahpasand, Khanjari, Abasali, Khani, Kalleghoochi and Akbari, were collected and moved to the laboratory. As the distribution of toxin and its dispersion is different in various parts of the kernel, the intensity of fungi contamination requires a homogeny and totally uniform sample to be studied. Thus, the nut kernels should be grinded. Then, a 10g of grinded kernel was added to 90 ml of 0.1% Pepton water and dilutions of 10^{-1} and 10^{-2} were prepared. In the next stage, 0.1 ml of the final concentration was cultured in specialized culture media with three replications. Then, the plates were incubated at 28 °C. Finally, *A. flavus* colonies were enumerated after 3-7 days. The grown fungi were identified by standard mycological techniques based macroscopic and microscopic morphology. For identification of *A. flavus* from other Aspergillus species, three medias, including CYA (Czapek Yeast Extract Agar), MEA (Malt Extract Agar) and AFPA (*Aspergillus flavus-parasiticus* Agar), were applied. AFPA is a selective identification medium for the detection of *A. flavus* group strains. Macromorphological features, which were considered for species identification and differentiation, included conidial and mycelial color, colony diameter, colony reverse color and the presence of sclerotia and cleistothecia. It is possible to distinguish these species from other Aspergillus based on the development of an orange color on the reverse of the plates. Also, colonies taxonomically between the two species (*A. flavus* & *A. parasiticus*) can be separated. Those of *A. flavus* were yellow-green in color and those of *A. parasiticus* were a distinctly darker green in color, referred to as near Ivy green. The colony diameter of *A. flavus* is 50 to 70 mm (Rodrigues *et al*., 2007). Also, aflatoxin B_1, B_2, G_1 and G_2 contents in different pistachio cultivars were analyzed by the HPLC method. In order to study the inhibitory effect of fungicides on growth of *A. flavus* and aflatoxin production in pre-harvest pistachio cultivars, the most contaminated cultivar, Owhadi, was selected, and a completely randomized design was carried out in field (*In vivo*). Two fungicides, tebuconazole 25% and mancozeb 80%, were applied at an application rate of 1 and 2 L or Kg ha^{-1}, respectively. Mancozeb is a fungicide in a subclass of carbamate pesticides called dithiocarbamates. I It is used to protect many fruit, nut and field crops from a wide spectrum of fungal diseases with the currency period of 7 days. Tebuconazole is a triazole fungicide used agriculturally to treat plant pathogenic fungi. It has a mode of action that is a systemic action (as well as preventive, curative, eradicative action) with the currency period of 14 days. In the orchards, three plots (in three repetitions) were considered, which included the first plot as a control group (not sprayed), a second plot (sprayed by tebuconazole 25%) and a third plot (sprayed by mancozeb 80%). The trees were sprayed by mancozeb every seven to ten days and every 14 days for tebuconazole. All conditions, including environment and farming operations, were considered the same in the plots. Finally, a composite sample was collected and moved to the laboratory. In the next step, pistachio kernels were grinded, and 10g of grinded pistachios were added to 90 ml of 0.1% Pepton water and a dilution of 10^{-2} was prepared. 0.1 ml of this concentration was cultured in a specialized culture medium (AFPA), and it was spread on the plate level (three times by random selection). Then, the plates were placed in an incubator for 28 °C. The number of colonies was counted after three to seven days. Aflatoxin B_1 and B_2 contents of the samples were analyzed using the HPLC method. The extract was placed in glass tubes and injected into the HPLC system. Standard solutions for the calibration curves were prepared on a daily basis. Determination of aflatoxins B_1 and B_2 were performed us-

ing HPLC with post-column fluorescence derivatization. A column coupled with a pre-column with the same stationary phase was used. HPLC analysis was carried out using isocratic elution. The system consisted of a pump coupled with a fluorescence detector, and the mobile phase consisted of water–methanol–acetonitrile.

Results

The results showed that there was a significant difference among the average number of colonies in different cultivars of pistachio. Table 1 shows the comparison of average contamination in 8 cultivars of Semnan province pistachio by *Aspergillus flavus* using Duncan statistical test. As it is seen in Fig. 1, among the tested parameters, Owhadi had the highest contamination, while Akbari had the lowest contamination.

Table 1. Comparison of average contamination in 8 cultivars of Semnan province pistachio by *Aspergillus flavus*.

Cultivar of pistachio	Average number of colonies	Duncan Statistical Classification
Owhadi	$7*10^3$	A
Ahmadaghaii	$6.366*10^3$	A
Shahpasand	$5.033*10^3$	B
Khanjari	$4.033*10^3$	C
Abasali	$1.333*10^3$	D
Khani	$0.633*10^3$	DE
Kalleghoochi	$0.5*10^3$	E
Akbari	$0.3*10^3$	E

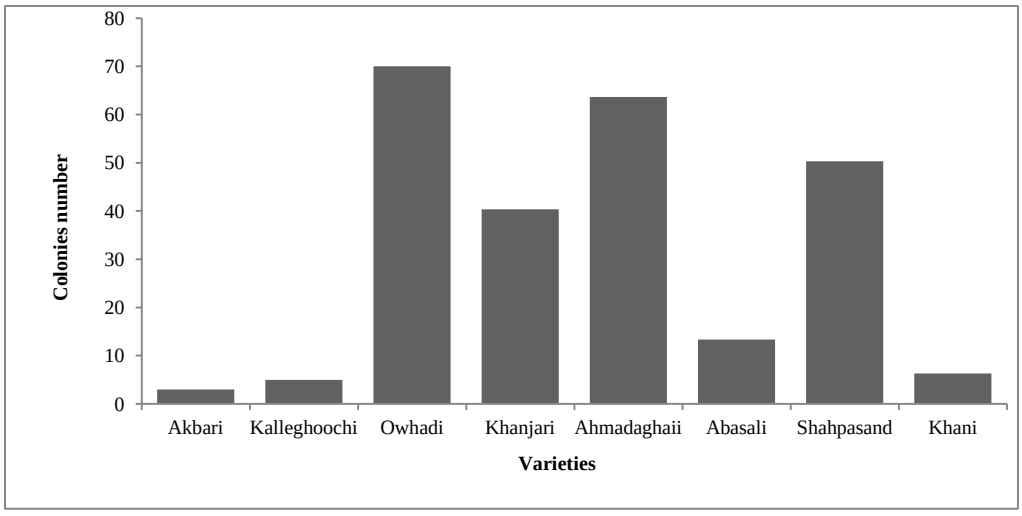

Fig. 1. The number of *A. flavus* colonies in different varieties of Semnan province pistachio

The study of aflatoxin and its measurement by HPLC method in different pistachio cultivars showed that among different cultivars, contents of B_1 and B_2 aflatoxins were observed in the Owhadi cultivar. The amount and type of aflatoxins in different pistachio cultivars are shown in Table 2. The amount of aflatoxins B_1 and B_2 in Owhadi cultivar were 0.35 ppb and 0.06 ppb, respectively. In Iran, the maximum tolerated levels

of aflatoxin in nuts and dried fruits are 8 ppb for aflatoxin B_1 and 15 ppb for total aflatoxins (B_1, B_2, G_1 and G_2).

Table 2. The amount and type of aflatoxin in 8 pistachio cultivars of Semnan province (2011-2012)

Pistachio variety	The amount of aflatoxin production (ppb)				
	B_1	B_2	G_1	G_2	Total
Abasali	ND	ND	ND	ND	ND
Shahpasand	ND	ND	ND	ND	ND
Owhadi	0.35	0.06	ND	ND	0.41
Kalleghoochi	ND	ND	ND	ND	ND
Akbari	ND	ND	ND	ND	ND
Khani	ND	ND	ND	ND	ND
Ahmadaghaii	ND	ND	ND	ND	ND
Khanjari	ND	ND	ND	ND	ND

ND= Not Detected

The inhibitory effect of two fungicides, including tebuconazole 25% and mancozeb 80%, on growth of *A. flavus* and aflatoxin production was assessed under *in vivo* conditions. The results are shown in Table 3 and Fig. 2. Table 3 shows the comparison of means by the Duncan method on effect of tebuconazole 25% and mancozeb 80% in the Owhadi variety. According to the results, tebuconazole 25% and mancozeb 80% reduced *A. flavus* growth compared to the control (at %5 level). Fig. 2 shows the effect of two fungicides on the average number of *A. flavus* mold colonies on the Owhadi variety.

Table 3. Comparison of means by Duncan on effect of tebuconazole 25% and mancozeb 80% in Owhadi variety

Variety	Experiment variant	Means	Duncan Statistical classification
Owhadi	Control	$7.26*10^3$	A
	Tebuconazole 25%	$6.33*10^3$	B
	Mancozeb 80%	$6.2*10^3$	B

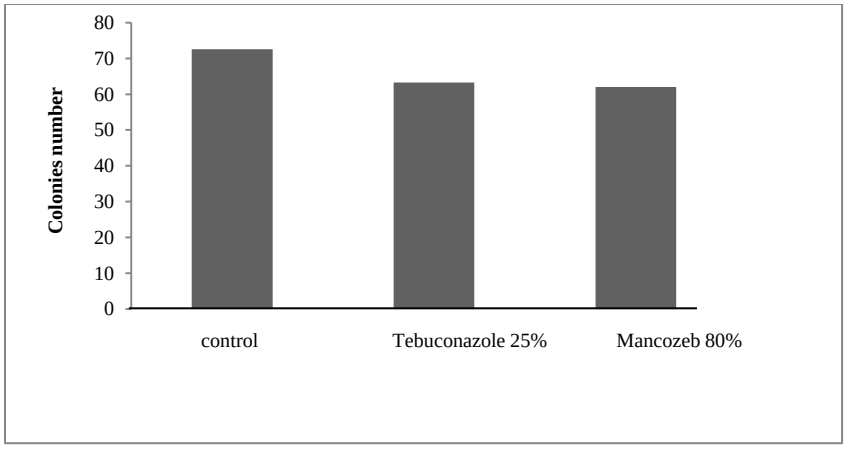

Fig. 2. Effect of two fungicides on average number of *A. flavus* mold colonies on Owhadi variety.

Aflatoxin B_1 and B_2 contents of the samples were analyzed using the HPLC method. The results are shown in Table 4. The amount of aflatoxins B_1 and B_2 in the Owhadi pistachio cultivar at specified concentration of

tebuconazole 25% were 0.34 ppb and 0.06 ppb, respectively. The amount of aflatoxins B_1 and B_2 at specified concentration of mancozeb 80% were 0.33 ppb and 0.05 ppb, respectively. The obtained results showed that tebuconazole 25% and mancozeb 80% reduced aflatoxin production at *in vivo* conditions. However, the reduction was not significant.

Table 4. The amount of aflatoxins B_1 and B_2 in Owhadi pistachio cultivar at specified concentrations of fungicides.

cultivar	Experiment variant	The amount of aflatoxin B_1 & B_2 (ppb)	
		B_1	B_2
Owhadi	Control	0.38	0.08
	Tebuconazole (25%)	0.34	0.06
	Mancozeb (80%)	0.33	0.05

Discussion

Our results showed that there was a significant difference among the average number of *A. flavus* colonies in different cultivars of pistachio. The Owhadi cultivar had the highest contamination while the Akbari cultivar had the lowest contamination. *A.flavus* produced B_1 and B_2 aflatoxins in the Owhadi cultivar. Research has shown that in Iran, pistachio nut contamination to *Aspergillus* species and their toxins are the most serious problems in pistachio production, consumption, and export processing. Aflatoxin producing *Aspergillus* species from pistachio were more prevalent in pistachio production regions (Rahimi *et al*., 2007; Houshyarfard *et al*., 2014). Houshyarfard *et al.* (2014) showed that five-hundred and eighty isolates were identified as *A. flavus* and it was the most abundant species of Aspergillus section *Flavi* in pistachio orchards of Iran. Mohammadi *et al.* (2009) reported that *A. flavus* was predominant in Iranian pistachio orchards. Magnoli *et al.* (1998) performed research on enumeration and identification of *Aspergillus* species in feeds from Argentina. The samples were examined for *Aspergillus* species. In addition, the capacity to produce aflatoxins by *Aspergillus* was determined. Their results showed that one of the most predominant species of *Aspergillus* was *A. flavus*, and 47% strains of *A. flavus* produced aflatoxins. Al-Gahtani *et al.* (2013) did an experiment to survey pistachio from three main regions in Saudi Arabia for the presence of *Aspergillus* spp. and to detect the levels of aflatoxin using the HPLC method. The study indicated that *A.flavus* showed the highest prevalence in the investigated samples. From fifteen samples, six isolates of *A. flavus* were positive for aflatoxin production.

Our results on the effect of two fungicides tebuconazole 25% and mancozeb 80% on growth of *A. flavus* and aflatoxin production in pistachio cultivar (Owhadi) showed that fungicides reduced *A. flavus* growth and aflatoxin production, but this reduction was not significant. The research of Brenneman *et al*. (1993) showed that diniconazole was not as effective in reducing aflatoxin contamination in peanuts, even at relatively high levels. Treatment with diniconazole had no effect on the *A. flavus* group in the soil or shells and reduced populations in seed only. One research study showed that natural plant products may successfully replace chemical fungicides and provide an alternative method to protect agricultural commodities from aflatoxin B_1 production by *A. flavus* (Krishnamurthy and Shashikala, 2006). Research has shown that essential oils from aromatic

plants inhibited the growth of *Aspergillus flavus* (Thanaboripat *et al*., 2007; Thanaboripat *et al*., 2004). Hence, it can be concluded that these natural plant products can be used instead of chemical fungicides, because they showed better results to control *A. flavus* growth and aflatoxin production. Several reports showed that the application of a biological control compared to a chemical control had better results in reducing growth and aflatoxin production by *A. flavus* (Brenneman *et al*., 1993). Conventional methods of *Aspergillus flavus* control with the use of fungicides were reported as ineffective when applied in environmentally safe concentrations. It is suggested that traditional control methods such as the use of pesticides, which effectively reduce populations of many plant pests in the field, have not been effective in controlling aflatoxin-producing fungi (Bhatnagar *et al*., 1993). These several conventional agronomic practices (such as use of fungicides) influence preharvest aflatoxin contamination of crops, but such procedures have only a limited potential for reducing aflatoxin levels in the field (Bhatnagar *et al*., 1993; Widstrom, 1987; Darrah and Bany, 1991; Lillehoj, 1991). These findings are supported by the results of this study.

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References

Al-Gahtani MF, Al-Othman MR, Mahmoud MA, Abdel-Aziz ARM (2013) Anti-aflatoxigenic effect of essential oils on *Aspergillus spp.* isolated from pistachio in Saudi Arabia. African Journal of Microbiology Research. 7(25), 3151-3159.

Aminshahidi M (1997) Study on aflatoxigenic *Aspergillus* species in native contaminated Pistachios of Iran and investigation of their capacity in producing aflatoxin. M.Sc. thesis, Microbiology group, College of Tehran University. 180p.

Bhatnagar D, Cotty PJ, Cleveland TE (1993) Preharvest aflatoxin contamination: molecular strategies for its control. Flavour and Safety: Molecular Analysis and Design (Spanier AM, Okai N, Tamura N, ed.). American Chemical Society, Washington D.C. 272–292.

Brenneman TB, Wilson DM, Beaver RW (1993) Effects of diniconazole on *Aspergillus* populations and aflatoxin formation in peanut under irrigated and nonirrigated conditions. Plant Disease. 77(6), 608-612.

Darrah LL, Bany BD (1991) Reduction of preharvest aflatoxin in corn. Pennington Center Nutrition Series, Mycotoxins, Cancer and Health; Eds; LSU Press: Baton Rouge, LA. 1, 283-310.

Formenti S, Naresh M, Pietri A, Battilani P (2012) *In vitro* impact on growth, fumonisins and aflatoxins production by *Fusarium verticillioides* and *Aspergillus flavus* using anti-fungal compounds and a biological control agent. Phytopathologia Mediterranea. 51(1), 247-256.

Houshyarfard M, Rouhani H, Falahati - Rastegar M, Malekzadeh - Shafaroudi S, Mehdikhani - Moghaddam E, Probst C (2014) Characterization of *Aspergillus* section *Flavi* from pistachio soils in Iran. Journal of Plant Protection Research. 54(4), 355-362.

Jalali L, Afshari H, Ashraf Sh, Ebadi AGh (2012) Study on the amount and type of aflatoxin produced by *Aspergillus flavus* in some pistachio cultivars of Khorasan-e-Razavi province. Journal of Pure and Applied Microbiology. 6(4), 1811-1816.

Khodavaisy S, Maleki A, Hossainzade B, Rezai S, Ahmadi F, Validi A, Rashidi A, Ghahramani E (2012) Occurrence of fungal contamination in pistachio and peanut samples from retail shops in Sanandaj, Iran. African Journal of Microbiology Research. 6(39), 6781-6784.

Krishnamurthy YL, Shashikala J (2006) Inhibition of aflatoxin B_1 production of *Aspergillus flavus*, isolated from soybean seeds by certain natural plant products. Letters in Applied Microbiology. 43(5), 469-74.

Lillehoj EB (1991) Aflatoxin: genetic mobilization agent. Mycotoxins in ecological systems. Eds. Marcel Dekker. Inc: NewYork, NY. 1-22.

Magnoli C, Dalcero AM, Miazzo R, Saenz MA (1998) Enumeration and identification of *Aspergillus* group and *Penecillium* species in poultry feeds from Argentina. Mycopathologia. 142, 27-32.

Mohammadi AH, Banihashemi Z, Haghdel M (2009) Identification and prevalence of *Aspergillus* species in soils of Fars and Kerman provinces of Iran and evaluation of their aflatoxin production. Rostaniha. 10(1), 8–30. [In Persian]

Onial P, Pandey A, Rawat MSM, Dayal R (2015) *In vitro* antifungal assessment of fruits pericarp of *Terminalia chebula* against fungus of forestry origin. International Journal of Herbal Medicine. 3(1), 52-55.

Rahimi P, Sharidnabi B, Bahar M (2007) *Aspergillus* species isolated from pistachio and determination of their aflatoxin production. Rostaniha. 8, 30–42. [In Persian]

Rahimi P, Sharifnabi B, Bahar M (2008) Detection of aflatoxin in *Aspergillus* species isolated from pistachio in Iran. Journal of Phytopathology. 156(1), 15–20.

Rodrigues P, Soares C, Kozakiewicz Z, Paterson RRM, Lima N, Venâncio A (2007) Identification and characterization of *Aspergillus flavus* and aflatoxins. Communicating Current Research and Educational Topics and Trends in Applied Microbiology. 527-534.

Santos L, Marin S, Sanchis V, Ramos AJ (2011) *In vitro* effect of some fungicides on growth and aflatoxins production by *Aspergillus flavus* isolated from Capsicum powder. Food Additives and Contaminants. 28(1), 98-106.

Thanaboripat D, Monkontanawut N, Suvathi Y, Ruangrattanametee V (2004) Inhibition of aflatoxin production and growth of *Aspergillus flavus* by citronella oil. KMITL Science Journal. 49(1), 1-8.

Thanaboripat D, Suvathi Y, Srilohasin P, Sripakdee S, Patthanawanitchai O, Charoensettasilp S (2007) Inhibitory effect of essential oils on the growth of *Aspergillus flavus*. KMITL Science and Technology Journal**.** 7(1), 1-7.

Wheeler MH, Bhatnagar D, Klich MA (1991) Effects of chlobenthiazone on aflatoxin biosynthesis in *Aspergillus parasiticus* and *A. flavus*. Pesticide Biochemistry and Physiology. 41(2), 190-197.

Widstrom NW (1987) Breeding strategies to control aflatoxin contamination of maize through host plant resistance. Aflaloxin in maize: A procudings of the workshop; CIMMYT: Mexico City, Mexico. Pp. 212-220.

Yabe K, Matsuyama Y, Ando Y, Nakajima H, Hamasaki T (1993) Sterochemistry during aflatoxin biosynthesis: Conversion of norsolorinic Acid to Averufin. Applied and Environmental Microbiology. 59, 2486-2492.