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Risk assessment of exposure to aflatoxin B1 and ochratoxin A through consumption of different Pistachio (*Pistacia vera* L.) cultivars collected from four geographical regions of Iran

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ABSTRACT

Iran is one of the main suppliers of pistachio for the European market accounting for over 90% of its demands; hence, efficient analytical methods are required for detection of mycotoxins contamination in pistachio kernels before exporting them. In this study, aflatoxin B1 (AFB1) and ochratoxin A (OTA) levels in five pistachio cultivars collected from four sites of Iran, were measured by HPLC. Based on the results, risk assessment for AFB1 and OTA residues was done. The highest mean concentrations of AFB1 and OTA were found in Ahmad-aghaei (4.33 and 2.19 ng/g, respectively) and Akbari (4.08 and 1.943 ng/g, respectively) cultivars from Rafsanjan, Iran. Even the highest concentrations of AFB1 and OTA in analyzed samples were lower than the corresponding maximum limits set by EU authorities. The hazard index (HI) value for consumers of Iranian pistachio is below one. It could be concluded that consumption of pistachio cultivated in these regions poses no health risk of mycotoxins exposure.

1. Introduction

Pistachio tree (*Pistacia vera*) belongs to the Anacardiaceae family. Pistachio kernels are often consumed as a snack (roasted and salted) and used in ice creams and confections. Strains of *Aspergillus flavus*, *Aspergillus parasiticus* and *Aspergillus nomius*, under certain conditions of temperature and humidity, can grow on crops such as nuts, cereals, cottonseed, corn, and produce mycotoxins and different carcinogenic secondary metabolites that may affect human health (Neamatallah and Serdar, 2013; Vlata et al., 2005).

These organisms grow under aerobic and anaerobic conditions (Nidhina et al., 2017). Detection of ochratoxin A (OTA) and aflatoxins (AFs) in food commodities is critical since their adverse effects on human health have been shown in several studies (Rastegar et al., 2017).

OTA has been found in various agricultural products, such as cereals including wheat, maize and barley, horticultural crops such dried vine fruits (Pfohl- Leszkowicz and Manderville, 2007; Polisenska et al., 2010), dried figs and dried apricots or all dried fruit (10 μg/kg), mixture of species (15 μg/kg), sunflower and pumpkin seeds, pistachios, hazelnuts or all tree nuts (5 μg/kg), liquorice placed on the market for the final consumer (10 μg/kg), herbs and herbal teas (10 μg/kg) and cocoa powder (2 μg/kg) (EFSA, 2017).

According to the Joint FAO/WHO Expert Committee on Food Additives, OTA exposure may cause neurotoxicity, immunotoxicity, nephrotoxicity and genotoxicity (Joint FAO/WHO, 2010) ; also,

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numerous studies correlated OTA with DNA damage, lipid peroxidation and cytotoxicity (Sorrenti et al., 2013).

According to The European Food Safety Authority limits, the maximum levels (MLs) for OTA in sunflower and pumpkin seeds, pistachios, hazelnuts or all tree nuts is 5 μg/kg and 10 μg/kg for liquorice placed on the market for the final consumer (EFSA, 2017).

Eighteen aflatoxins are known, but only AFB1, AFB2, AFG1 and AFG2 have been found in agricultural crops (Torre et al., 2015). Among them, aflatoxin B1 (AFB1) is the most toxic metabolite (Tsakiris et al., 2013). The International Agency for Research on Cancer (IARC) has also noted that AFB1 as a lipophilic molecule, accumulates in the liver inducing acute hepatitis, that following long-term exposure, may result in liver cancer [(available at http://monographs.iarc.fr/ENG/Classification/List_of_Classifications.pdf and http://monographs.iarc.fr/ENG/Classification/)]. However, so far, no carcinogenic properties have been demonstrated for AFB2, AFG1 and AFG2 in humans (Joint FAO/WHO, 2001). Also, for other mycotoxins (e.g. zearalenone), carcinogenicity and teratogenicity have been reported (Alegakis et al., 1999; Vlata et al., 2006). According to the IARC, OTA and aflatoxins have been classified as “possibly carcinogenic to humans (Group 2B)” and “carcinogenic to humans (Group 1)”, respectively (available at http://monographs.iarc.fr/ENG/Classification/List_of_Classifications.pdf and http://monographs.iarc.fr/ENG/Classification/).

The volume of food consumed over a specified period of time and the level of xenobiotic residues are the two critical parameters required for risk assessment. According to the risk assessment principals, the estimated human exposure to xenobiotics should be compared with the reference values such as Acceptable Daily Intake, Acute Reference value, etc. In case of genotoxic carcinogens such as AFB1, the non-threshold approach of “as low as reasonably achievable” (ALARA) (EFSA, 2007a), or the Margin of Exposure (MoE) approach are usually used (EFSA, 2007b)

Determination of AFB1 levels, along with elimination of AFB1 or reduction its concentration in pistachio have attracted considerable attention in major pistachio-producing countries such as Iran. During cultivation period, depending on the weather conditions or/and insufficient plant protection practices, pistachios may become contaminated with mycotoxins produced by fungi. During storage, water activity (a_w) and temperature crucially affect fungal growth and aflatoxins production (Liu et al., 2017). Traditional harvesting and processing methods combined with storage under high humidity and temperature, favor microorganisms development. Moreover, soil, animals, insects and humans are other potential sources of contamination (Campbell et al., 2003).

Aflatoxin contamination also depends on the form of pistachio shell, which is related to cultivar type. Early splitting and hull cracking are two main factors allowing the entrance of fungus into the pistachio (Tajabadipour and Sheibani Tezerji, 2011). In early summer, hull is attached to shell and then shell splits. Shell splitting facilitates the consumption of pistachio for consumers, but the hull does not split and protects the kernel against penetration of insects and fungi. In some pistachio cultivars known as "early split", hull splitting can increase the chance of aflatoxin contaminations (Doster and Michailides, 1995).

The European Food Safety Authority (EFSA) has set the MLs of 4 μg/kg for total aflatoxins (B1 + B2 + G1 + G2) in groundnuts, nuts, dried fruits, cereals and processed products and 2 μg/kg for AFB1 (EFSA, 2007b). Moreover, the EFSA has established an ML of 5 μg/kg for OTA (EFSA, 2017).

This study aimed to (1) measure AFB1 and OTA levels in five pistachio cultivars collected from four sites of Iran, (2) assess the health risk posed by consumption of pistachio based on hazard index (HI), and (3) introduce the best cultivar and site of cultivation in which the aflatoxin content is near the standard levels.

2. Materials and methods

2.1. Chemicals and reagents

Aflatoxin B1 and ochratoxin A with 98% purity were procured from Sigma (St. Louis, MO, USA). Ethanol, methanol, acetonitrile, phosphate-buffered saline (PBS) were supplied from Sigma (Sigma-Aldrich, Steinheim, Germany). Solvents were filtered and degassed prior to application. All other chemicals were of analytical grade and supplied by Merck (Darmstadt, Germany) and Sigma (St. Louis, MO, USA). Immunoaffinity columns (IAC) for AFB1 and OTA were prepared from Libios (Pontcharra-Sur-Turdine, France).

2.2. Plant materials and climatic and topographic characteristics

Five ripe commercial pistachio cultivars namely, Akbari, Ahmad aghaei, Badami-e-sefid, Kalehghoochi and Owhadi, were collected from Rafsanjan, Damghan, Sarakhs and Feizabad as the main sites of pistachio cultivation in Iran (Esfandiyari et al., 2012).

The samples were dried and stored in polyethylene bags at −18 °C until chemical analysis. Climatic and topographic characteristics of cultivation sites are shown in Fig. 1 and Table 1.

2.3. Mycotoxins analysis

2.3.1. Determination of Aflatoxin B1 (AFB1) levels

Dried kernels were grounded using a mechanical blender. First, AFB1 standard solution was prepared. Then, 5 g sodium chloride and 300 mL of a water-methanol (8:2 v/v) solution, were added to 50 g of each pistachio sample. To obtain a homogenous suspension, all mixtures were separately blended 2000 RPM) for 5 min, then passed through a filter paper (Whatman No. 4). Next, 10 mL of each pistachio sample extract was diluted with 60 mL of phosphate-buffered saline (PBS) solution and passed through an IAC. The column was incubated at room temperature to stabilize and further conditioned using 10 mL of PBS, at a flow rate of 1 mL/min under the gravity of methanol (1.5 mL, flow rate of 1 mL/min) to elute AFB1 from IAC. Then, the dried residue was dissolved in methanol and stored in a vial for determination of AFB1 using HPLC (Janati et al., 2012).

2.3.2. Determination of Ochratoxin A (OTA) levels

Based on the method described by Institute of Standards and Industrial Research of Iran (Heshmati and Mozaffari Nejad, 2015), 50 g of each pistachio sample either non-spiked or spiked with an specific volume of OTA stock solution, was mixed with 750 mL of distillated water and blended using a mechanical blender at 2000 RPM for 5 min. Then, samples were precisely weighed (50 g), and mixed with 300 mg sodium bicarbonate and 60 mL acetonitrile and left for 5 min. Afterwards, samples were filtered through a filter paper (Whatman No. 4) and 10 mL aliquots of each sample was mixed with 40 mL PBS solution. Then, 40 mL of solution was passed through an IAC, which was conditioned by 20 mL PBS at a flow rate of 1 ml/min under gravity, before use at room temperature. Then, the columns were washed with 10 mL distilled water at a flow rate of 1 mL/min and dried. OTA was eluted twice from the IAC using methanol: acetic acid (98:2, v/v) solution with a vacuum. Eventually, 20 μL eluate was injected to the HPLC (Heshmati and Mozaffari Nejad, 2015).

2.4. HPLC analysis

The analyses of AFB1 and OTA were conducted by a reverse-phase HPLC coupled with fluorescence detection (HPLC-FD) (Waters Corporation, Milford, MA, USA) and equipped with a PR C18 analytical column (250 mm × 4.6 mm, i.d., 5 μm). The column oven was set at 25 °C. The mobile phase utilized for AFB1 determination, consisted of water: methanol: acetonitrile in a ratio of (60:20:10 v/v/v) and used at

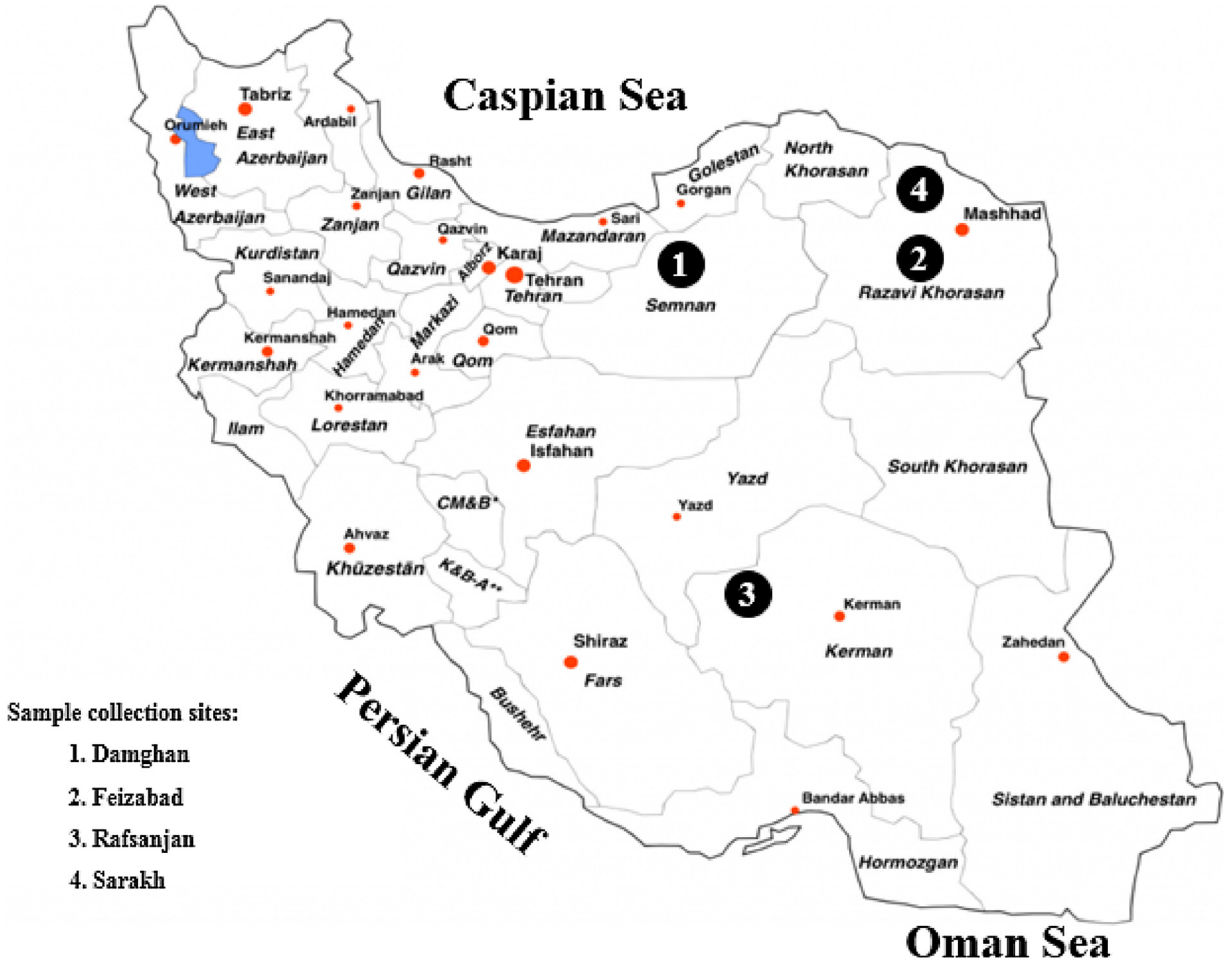

Fig. 1. Cultivation zones from where the samples were collected.

a flow rate of 1 mL/min; also, the wavelengths of excitation and emission were 365 and 435 nm, respectively. Furthermore, for OTA determination, a mobile phase composed of acetonitrile: water: methanol: acetic acid in a ratio of (39:30:30:1 v/v/v/v) was used at a flow rate of 1 mL/min and the wavelengths of excitation and emission were 333 and 477 nm, respectively.

2.5. Calibration curve

A calibration curve (Table 2) was prepared by using the standard solutions at 0.312, 0.625, 1.25, 2.50, 5.00, and 10.0 ng/ml for AFB1 and 2.50, 5.0, 10.0, and 20.0, 40.0 ng/ml for OTA. The calibration curve was constructed before analysis of the samples, the linear regression equations were used for AFB1 and OTA measurement in pistachio samples.

Table 2
Calibration curve plotted for the mycotoxins.

Mycotoxin	Equation of calibration curve	R^2
AFB1	Y = 12728X + 146	0.993
OTA	Y = 15299X + 3792	0.997

2.6. Human risk assessment of exposure to AFB1 and OTA via consumption of selected pistachio cultivars

2.6.1. Exposure estimation

The EDI (Estimated Daily Intake) was calculated using the mean level of AFB1 and OTA in pistachio samples, the daily pistachio intake (0.82 g/person/day) (Taghizadeh et al., 2017), and the average body weight (70 kg/person). The EDI for AFB1 and OTA was calculated according to the following formula and expressed in ng/kg of body weight/day (ng/kg b.w. / day) (dos Santos et al., 2013).

Table 1
Geographical location, topographic and climatic characteristics of the study stations.

Station	Latitude (N)	Longitude (E)	Altitude (m)	Mean annual temperature (°C)	Mean annual precipitation (mm)	Average of relative humidity (%)
Rafsanjan	30°41′	55°9′	1580.9	15	105	29
Damghan	36°04′	54°25′	1180	13	127	55
Sarakhs	36°32′	61°10′	235	17.9	189.6	49
Feizabad	35°01′	58°78′	940	28.65	20	26

$$EDI = \frac{daily\ pistachio\ intake \times mean\ level\ of\ \text{AFB1 or OTA}}{average\ body\ weight}$$

2.6.2. Estimation of Hazard Index (HI)

According to the below mentioned formula, the Hazard Index (HI) was calculated by dividing the EDI by TD_{50}, divided by a safety factor of 50,000. TD_{50} is the dose (mg/kg/body weight/day) required to induce tumors in half of test animals that would have remained tumor-free at zero dose (Ishikawa et al., 2016; Ismail et al., 2016; Tsakiris et al., 2013).

$$HI = \sum_{n=0}^{i} \frac{(EDI/TD50)}{50000}$$

2.7. Statistical analysis

All measurements were carried out in triplicate by JMP 8 (SAS Campus Drive, Cary, NC 27513) and Excel software. All data were expressed as mean ± standard deviation. Differences among mean values were evaluated by two-way analysis of variance (ANOVA) and significant intergroup differences and Least Significant Difference Test (LSD, and Student's *t*-test. A p-value < 0.05 was considered statistically significant.

3. Results

3.1. Method validation

The recovery value for AFB1 and OTA was calculated by analyzing the AFB1 and OTA concentrations in the spike samples and comparing them with those of standard solutions (2, 4, and 6 ng/g for AFB1 and 4, 8, and 12 ng/g for OTA solutions). All the experiments were performed in triplicate. Limit of detection (LOD) for AFB1 and OTA were 0.066 and 0.27 ng/g, respectively, and limit of quantification (LOQ) for AFB1 and OTA were 0.2 and 0.81 ng/g, respectively, (Table 3). In this study, we determined the levels of AFB1 and OTA in 600 samples; based on our results, 270 samples were positive for AFB1 and 180 samples were positive for OTA.

3.2. OTA and AFB1 mean values in pistachio samples

According to our results, both the area of cultivation and the harvesting techniques significantly affected AFB1 and OTA concentrations in pistachio samples (Table 4).

Among different cultivation areas and cultivar types, the maximum AFB1 mean values were recorded for Ahmad aghaei cultivar collected from Rafsanjan cultivation site, followed by Akbari (collected from Rafsanjan) and Ahmad aghaei (collected from Feizabad) and the minimum concentrations of AFB1 were found in Kalehghoochi cultivar collected from Sarakhs cultivation site. In terms of OTA content, the maximum concentrations were found in Ahmad aghaei (collected from Rafsanjan) and the minimum levels were reported in Ahmad aghaei (collected from Damghan) and Akbari (collected from Feizabad). As shown in Table 4, in all cultivation sites, AFB1 and OTA in all Owhadi and Badami-e-sefid samples were found at trace levels or were "not detected".

Table 3
Limit of detection (LOD) and limit of quantification (LOQ) for AFB1 and OTA as measured by HPLC-FD in this study.

AFB1	LOD	0.066 ng/g
	LOQ	0.20 ng/g
OTA	LOD	0.27 ng/g
	LOQ	0.81 ng/g

LOD: Limit of detection.
LOQ: Limit of quantification.

3.3. Human risk assessment of exposure to AFB1 and OTA via consumption of selected pistachio cultivars

3.3.1. Estimated daily intake (EDI)

The EDIs calculated for AFB1 and OTA for Iranian people via consumption of selected pistachio cultivars are presented in Table 5.

3.3.2. Hazard index (HI)

Generally, it is accepted that an HI ≤ 1 indicates no significant health risk. However, the possibility of long-term adverse health effects increases with increasing HI values as an HI between 1.1 and 10 reflects a moderate risk (Lemly, 1996), and HI < 10 indicates high risk (Ogunkunle et al., 2013). HI value for exposure to AFB1 and OTA via consumption of collected pistachio cultivars consumed by the Iranian population, is less than one. The aforementioned values imply that intake of pistachios from these cultivars most likely does not pose risk to Iranian population health.

4. Discussion

In our study, the mean AFB1 level was 1.14 ng/g, which was lower than the modified maximum level of AFB1 in pistachio kernels (2 ng/g) proposed for direct human consumption. The mean level of OTA content was 0.413 (ng/g), which was also below the level established by the European Union for OTA (i.e. 5 ng/g) (EFSA, 2007a, 2007b). In this experiment, aflatoxin contamination was only observed in pistachios with discolored shell. In our study, in all cultivation sites, the highest content of AFB1 was found in Ahmad aghaei pistachio cultivar which was significantly higher compared to other cultivars ($P < 0.05$). In a previous report, consistent with our results, Owhadi cultivar had the lowest aflatoxin content which was related with shell discoloration (Tajabadipour and Sheibani Tezerji, 2011).

In another study, AFB1 levels varied among the evaluated cultivars. However, Owhadi pistachio cultivar showed contamination levels lower than the AFB1 tolerable limit proposed by European Commission (Bensassi et al., 2010).

In the present experiment, in Owhadi cultivar, AFB1 was not detected or trace amounts were found (i.e. between LOD and LOQ values). AFB1 levels were markedly related to the type of cultivar as it was shown that some cultivars might be more susceptible to AFB1 contamination (Dowd et al., 2005). Based on the literature, mature pistachio cultivar was the most susceptible type towards AFB1 contamination, which is seemingly due to the invasion of *A. flavus* rather than *A. parasiticus* to this cultivar. In previous studies, a positive correlation between *A. flavus* growth and AFB1 content of pistachio (Ghali et al., 2009). Early splitting and hull cracking are other main reasons favoring contamination.

Mostly, early-splitting pistachio cultivars like Ahmad ahghaei and Akbari, are frequently contaminated with molds and insects. Early-splitting pistachios are more susceptible to *Aspergillus* spp infection. Our results corroborate the differences among various geographical regions regarding mycotoxin contaminations. Contamination may occur in the orchard, or following long rains and insufficient drying during harvest which provide appropriate conditions for *Aspergillus* growth and mycotoxin production (Bensassi et al., 2010). AFB1 contamination in pistachio nuts also depends on post-harvest and storage conditions; for example, high temperatures and humidity may induce fungi growth (Iqbal et al., 2006). Orchard management is the other main factor which could potentially lead to contamination. There is a positive correlation between harvest delay and increase in *Aspergillus* contamination.

Aflatoxins (AF) and ochratoxin are the most-studied mycotoxins as their presence in foodstuff may pose a great danger to humans health

Table 4
Levels of AFB1 and OTA in five different pistachio cultivars collected from four cultivation sites in Iran.

Site of cultivation	Cultivar type	Range AFB1 (min-max) (ng/g)	AFB1 mean value (ng/g)	Range OTA (min-max) (ng/g)	OTA mean value (ng/g)
	Ahmad aghaei	0.31-3.844	2.077 ± 0.69[g]	0.922-1.02	0.971 ± 0.3[e]
	Akbari	0.22-3.676	1.948 ± 0.54[h]	LOQ	LOQ
Damghan	Kalehghoochi	0.525-2.729	1.627 ± 0.53[i]	LOQ	LOQ
	Owhadi (Fandoghi)	< LOD	< LOD	< LOD	< LOD
	Badami-e-sefid	< LOD	< LOD	< LOD	< LOD
	Ahmad aghaei	0.481-7.245	3.863 ± 1.26[c]	0.958-1.394	1.176 ± 0.4[c]
	Akbari	0.310-5.852	3.081 ± 0.003[e]	0.886-1.044	0.965 ± 0.32[e]
Feizabad	Kalehghoochi	0.552-3.734	2.143 ± 0.004[f]	LOQ	LOQ
	Owhadi (Fandoghi)	LOQ	LOQ	< LOD	< LOD
	Badami-e-sefid	LOQ	LOQ	< LOD	< LOD
	Ahmad aghaei	0.701-7.959	4.33 ± 0.005[a]	1.062-3.318	2.19 ± 0.002[a]
	Akbari	0.592-7.568	4.08 ± 0.005[b]	1.001-2.885	1.943 ± 0.003[b]
Rafsanjan	Kalehghoochi	0.484-6.422	3.453 ± 0.005[d]	0.933-1.127	1.03 ± 0.004[d]
	Owhadi (Fandoghi)	LOQ	LOQ	< LOD	< LOD
	Badami-e-sefid	LOQ	LOQ	< LOD	< LOD
	Ahmad aghaei	0.576-1.346	0.961 ± 0.005[j]	LOQ	LOQ
	Akbari	0.282-0.692	0.487 ± 0.005[k]	LOQ	LOQ
Sarakhs	Kalehghoochi	LOQ-0.286	0.233 ± 0.003[l]	LOQ	LOQ
	Owhadi (Fandoghi)	< LOD	< LOD	< LOD	< LOD
	Badami-e-sefid	< LOD	< LOD	< LOD	< LOD

Data are expressed as mean of AFB1 and OTA content (ng/g) of samples ± SD (standard deviation) of three replicates.
LOD: Limit of detection.
LOQ: Limit of quantification.

Table 5
Estimated daily intake (EDI) for AFB1 and OTA for Iranian people via consumption of selected pistachio cultivars.

Type of mycotoxin	EDI (ng/kg bw/day)
AFB1	0.013
OTA	0.004

(Ishikawa et al., 2016).

In epidemiological studies on OTA effects, there were some evidence linking human diseases to OTA contamination. In this respect, OTA is considered to be correlated with kidney diseases; therefore, exposure to OTA via dietary intake could be regarded as a potentially serious risk (Bui-Klimke and Wu, 2015). Risk assessment is used to determine the health effects of mycotoxin exposure and guide food regulators to set thresholds for these chemicals in foodstuffs (Kuiper-Goodman, 1995). Iran is the main exporter of pistachios to Europe; therefore, regular monitoring of pistachio nuts is highly important to control their safety and minimize mycotoxins contamination. Our data, consistent with a previous study (Van de Perre et al., 2015), showed that exposure to AFB1 and OTA intake via pistachio consumption was lower than limits set by the European authorities. Reports indicated that exposure to high levels of OTA occurred following consumption of only one pistachio cultivar (with an OTA level of 821 ng/g) in the United States; nonetheless, considering the population, this affected only 1 percent of individuals consuming this pistachio cultivar. Hence, due to the infrequency of exposure to high levels of OTA via pistachio intake, along with low pistachio consumption in US, OTA poisoning following pistachio consumption, is unlikely (Mitchell et al., 2017).

An evaluation in Swedish population revealed that the mean dietary intake of aflatoxins was up to 1750 µg/ person.day for groundnuts and 3.5 µg/ person.day for almonds. It was shown that in ten percent of nut samples, AFT levels were higher than the maximum limits set by the EU (Thuvander et al., 2001). AFs and OTA in 88 samples including dried mulberry, date, fig and apricot collected from the Iranian market, were measured and the results showed that, the exposure to OTA via consumption of dried fruits did not exceed PTWI (Heshmati et al., 2017), which was consistent with our results.

In our study, based on the level of pistachio consumption, we showed that the estimated daily intake of AFB1 and OTA through consumption of this product is lower than the maximum limits recommended by the European Commission. Also, calculation of the HI value indicated that consumption of these selected pistachio cultivars, poses no health risk to Iranian consumers.

5. Conclusion

Measurement of the levels of two mycotoxins in five commercial pistachio cultivars collected from four main cultivation sites of Iran, showed that in all samples, AFB1 and OTA levels were below the maximum limit recommended by the European Commission regulations. Moreover, the HI value was below 1 which indicates that consumption of these cultivars pose no significant health risk to humans. It is highly suggested to conduct similar studies on other nuts cultivated in Iran and exported to other countries.

Conflict of interest

The authors declare no conflicts of interest.

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