



Article

Seasonal Variation in Aflatoxin Levels in Edible Seeds, Estimation of Its Dietary Intake and Vitamin E Levels in Southern Areas of Punjab, Pakistan

Ahmad Faizal Abdull Razis ^{1,2,*} , Muhammed Muzammel Shehzad ³, Sunusi Usman ¹ ,
Nada Basheir Ali ², Shahzad Zafar Iqbal ^{3,*} , Nadia Naheed ³ and Muhammad Rafique Asi ⁴

- ¹ Natural Medicines and Products Research Laboratory, Institute of Bioscience, Universiti Putra Malaysia (UPM), Serdang 43400, Selangor, Malaysia; usunusi.bch@buk.edu.ng
- ² Department of Food Science, Faculty of Food Science and Technology, Universiti Putra Malaysia (UPM), Serdang 43400, Selangor, Malaysia; nada44basher@gmail.com
- ³ Department of Applied Chemistry, Government College University Faisalabad, Faisalabad 38000, Pakistan; muzammelshehzad786@gmail.com (M.M.S.); eruditenadia@gmail.com (N.N.)
- ⁴ Food Toxicology Lab, Nuclear Institute for Agriculture and Biology (NIAB), Faisalabad 38000, Pakistan; asimhammad@yahoo.co.uk
- * Correspondence: madfaizal@upm.edu.my (A.F.A.R.); shahzad10542005@yahoo.com (S.Z.I.); Tel.: +60-397693073 (A.F.A.R.); +92-300-7684577 (S.Z.I.)

Received: 25 October 2020; Accepted: 19 November 2020; Published: 2 December 2020



Abstract: A total of 779 samples of edible nuts (melon seeds, watermelon seeds, pumpkin seeds, and cantaloupe seeds) from Southern Punjab (Pakistan), were collected during the summer and the winter seasons. The natural occurrence of aflatoxins (AFs) and vitamin E (tocopherols) levels were investigated using HPLC. The results have shown that 180 (43.4%) of samples from the winter season and 122 (33.4%) samples from the summer season were found positive for AFs. Elevated average levels of total AFs (20.9 ± 3.10 $\mu\text{g}/\text{kg}$, dry weight) were observed in watermelon seeds without shell, and the lowest average amount (15.9 ± 3.60 $\mu\text{g}/\text{kg}$) were documented in melon seeds without shell samples from the winter season. An elevated average amount of total AFs 17.3 ± 1.50 $\mu\text{g}/\text{kg}$ was found in pumpkin seeds available without a shell. The results have documented a significant difference in total AFs levels in edible seeds available with shells versus without shells ($\alpha = 0.05$ & 0.01). The highest dietary intake of 6.30 $\mu\text{g}/\text{kg}/\text{day}$ was found in female individuals from consuming pumpkin seeds (without shell) in the winter season. A value of 3.00 $\mu\text{g}/\text{kg}/\text{day}$ was found in pumpkin seed without shell in the summer season in female individuals. The highest total tocopherol levels were 22.2 ± 7.70 $\text{ng}/100$ g in pumpkin seeds samples from the winter season and 14.5 ± 5.50 $\text{mg}/100$ g in melon seed samples from the summer season. The variation of total tocopherol levels in edible seeds among the winter and summer seasons showed a significant difference ($p \leq 0.0054$), except watermelon seeds samples with non-significant differences ($p \geq 0.183$).

Keywords: AFs; edible seeds; tocopherols levels; dietary intake

1. Introduction

In recent years, owing to their beneficial nutritional value linked with the management of acute diseases such as cardiovascular conditions, cancer, obesity, and diabetes, the use of seeds as a dietary additives has increased substantially [1,2]. The functional and nutraceutical properties are focused on their high content of essential proteins, fatty acids, synthetic fibers, antioxidants, carotenoids, minerals, and vitamins [2]. Seeds of the Cucurbitaceae family (including watermelon or pumpkin) are mostly thrown out, however, they can be utilized for food enrichment or nutraceutical development [3].

Herbaceous plant chia seeds are considered as one of the most nutritious foods due to their bioactive peptides and proteins [4]. The majority of edible seeds are usually consumed as vegetable oils [5]. The seeds of pumpkin, winter melon, and watermelon are huge, plentiful, and edible, and pumpkin seeds are roasted with relish in some geographic areas [6]. Charmagaz (watermelon seeds, melon seeds, cantaloupe seeds, and pumpkin seeds) are extremely popular in Pakistan and believed to help in brain development and rejuvenation. They are mostly used to make different types of drinks such as sardie (a cold drink) and make sweet dishes, smoothies, consumed in summer and halwa.

The edible seeds may be affected due to bad weather conditions and poor pre-harvest or post-harvest conditions. The drying, storage, and transportation processes are critical and may contribute to fungal attacks [7]. Insect and pests are the main cause of damage, but molds are frequently identified as major contributors to agricultural goods' damage during storage [8]. These fungi produce mycotoxins, which may have lethal and carcinogenic impacts on human and animal health [9].

Mycotoxins are recognized as naturally occurring secondary metabolites produced by filamentous fungi with various chemical structures [10,11]. The Food and Agricultural Organization (FAO) reported that globally some 25 percent of crops are infected with mycotoxins [12,13]. Major classes of mycotoxins with highest influence on human beings and agriculture losses are aflatoxins (AFs), ochratoxin A (OTA), zearalenone (ZEA), deoxynivalenol (DON), and fumonisin (FB) [14,15]. However, nuts and seeds are mostly contaminated with aflatoxins [16]. The most studied class of AFs is a group of compounds formed primarily by *Aspergillus flavus* and *Aspergillus parasiticus* [17,18]. Previous studies have shown that 20 different types of AFs are known, but the most commonly known classes are AFB₁, AFB₂, AFG₁, and AFG₂ [19,20]. Health effects like teratogenicity, mutagenicity and hepatocarcinogenicity are caused due to the fact these toxins most attack the liver [21]. The International Agency for Research on Cancer (IARC) has classified AFs as carcinogenic to humans (Group 1). The relative toxicity ranking of the various forms of AFs is AFB₁ > AFG₁ > AFB₂ > AFG₂ [22]. The most important and deeply studied class of AFs is AFB₁ due to its high toxicity and widespread occurrence in various staple foods and feeds documented in previous studies. Almost 119 countries have established regulations for AFs in international trade and defined their regulatory limits [23]. The maximum acceptable limits for total aflatoxins (AFB₁ + AFB₂ + AFG₁ + AFG₂) are 15 µg/kg for hazelnuts, groundnuts, Brazilian nuts, apricot kernels, almonds, and pistachios for food practices [24]. However, no regulations are implemented for these toxins by Pakistan.

Vitamin E, commonly occurring in various foods like edible oils, cereals, margarine, nuts, fatty fishes, and egg yolk, contains numerous essential lipophilic organic compounds necessary to maintain the human's smooth functions body. It is also used as food additives because of their antioxidants properties and their ability to protect against fat rancidity [25]. Some studies have documented that, antioxidants were found effective in inhibiting or control aflatoxigenic fungi and the production of AFB₁ and fumonisins in stored maize [26,27].

The environmental conditions of Pakistan are favorable for fungal propagation and, consequently, for the production of AFs [28]. The seasonal variation may affect aflatoxins contamination during storage or selling in the supermarket or open shops. The humid environment may increase moisture level and provide favorable conditions for fungal growth. In our previous studies, a considerable amount of AFs in dry fruits was documented (highest levels of total AFs 7.30 ± 1.80 µg/kg in peanuts without shell, the lowest mean of 2.90 ± 1.50 µg/kg in watermelon seeds with shell samples) [29,30]. The high amounts of AFs were observed in peanut and peanut products [31]. Therefore, the present research is aimed to examine; the natural presence of AFs levels in edible seeds, to compare the amounts of AFs in edible seeds with the European Union (EU) recommended limits, to determine the dietary intake of AFs in the local population, and to investigate the vitamin E content in selected edible seeds. The findings of the current work will be beneficial for farmers, consumers, and traders to create awareness about the health risks related to these toxins, e.g., to purchased edible seeds in proper packing and the seeds available in shells have fewer levels as compared to without shell samples.

2. Materials and Methods

2.1. Sampling

A total of 779 samples of edible seed (melon seeds, watermelon seeds, pumpkin seeds, and cantaloupe seeds) samples (414 from the winter season and 365 from the summer season) were collected from various cities in Southern Punjab (Multan, Bhakkar, Layyah, and Muzaffargarh) Pakistan. The seeds samples were directly purchased from the market, open shops, and a superstore. The edible seed samples were obtained randomly. The terms of seasons, i.e., the summer season, is comprised of (May 2019 to August 2019) and the winter season (November 2019 to January 2020). The deshelling of edible samples was done from a market, where samples were purchased. The sample size of each seed was kept at 1 kg, and the samples were ground in fine particle size with a grinding mill (Retsch, Dusseldorf, Germany). The mill was cleaned properly after grinding each sample. After grinding, the samples were stored in plastic polyethylene bags and placed in the laboratory (Food Safety and Food Toxicology, Department of Applied Chemistry, GCUF) at $-20\text{ }^{\circ}\text{C}$ in a freezer.

2.2. Chemicals and Reagents

Aflatoxin standards (in acetonitrile $1\text{ }\mu\text{g/mL}$) and chemicals like methanol (HPLC grade), chloroform (HPLC grade), cupric carbonate (HPLC grade), anhydrous sodium sulfate (HPLC grade) were acquired from (Sigma-Aldrich, Steinheim, Germany). The other chemicals and reagents used in the current research were of high purity grade ($\geq 90\%$), and the double-distilled water was used for analysis.

2.3. Aflatoxins Extraction

The extraction of AFs from edible seeds was carried out following the method of Schuller and Van Egmond [32] with some modifications. The edible seeds were kept at $40\text{ }^{\circ}\text{C}$ for dryness in a vacuum oven and then milled. Then, 40 g of dried ground edible seeds were taken and added in 200 mL of methanol/water (80:20 *v/v*) solution. Afterward, 5 g of NaCl was added to this mixture and blended for 2 min. Whatman no.1 filter paper was used to filter the mixture. After carrying out the filtration, 50 mL of the filtrate was taken in a separatory funnel, and 50 mL of chloroform was added, shaken gently for 1 min on the shaker, and left to develop phases. Then 5 g of cupric carbonate was taken in a beaker, and the aqueous chloroform layer was separated and filtered using anhydrous sodium sulfate. The chloroform extract was taken in a vial and dried using a nitrogen stream with controlled temperature ($50\text{ }^{\circ}\text{C}$) after filtration. The samples were derivatized by adding 100 μL of trifluoroacetic acid (TFA) to 400 μL of mixture of samples and then vortexed for 30 s on vortex (Scilogex SCI-FS, Rocky Hill, CT, USA), and allowed the mixture to stand for 15 min. Lastly, the solution of 900 μL (water/acetonitrile) with a ratio of (9:1 *v/v*) was added and vortexed for 30 s and subjected for HPLC analysis.

2.4. Extraction of Oil

The extraction of oil from each edible seed was determined following the method of Gliszczynska-Swigło et al. [33]. In a cotton thumb bell, 20 g of ground sample was taken, and soxhlet apparatus (at $110\text{ }^{\circ}\text{C}$, for a duration of 24 h) was used with 250 mL of n-hexane to extract the oil of edible seed sample. Approximately (0.04 to 0.12 g) of the sample was dispersed in 1 mL of 2-propanol and vortexed for 2 min and stored in a vial for HPLC analysis.

2.5. HPLC Conditions

The analysis of AFs and tocopherols were performed on an HPLC instrument (Model-LC-10A, Shimadzu, Kyoto, Japan) equipped with a C_{18} column (250 mm \times 4.6 mm, 5 μm) (Discovery HS, Bellefonte, PA, USA) provided with a fluorescence detector (RF-530). The mobile phase for tocopherols

has consisted of 50% acetonitrile (solvent A) and 50% methanol (solvent B) with a 1 mL/min flow rate and a 20 µL of injection volume. The fluorescence detector was set at 325 nm of an emission wavelength and 295 nm of excitation wavelength. The flow rate of 1.5 mL/min of isocratic mobile phase with the composition of (water/methanol/acetonitrile) (60:20:20 *v/v/v*) was used for the analysis of AFs. The emissions and excitation wavelengths were 440 and 360, respectively. The final injection volume was 20 µL.

2.6. Dietary Intake Evaluation

According to the formula used by FAO/WHO [34], the estimated daily intake (EDI) can be calculated as:

$$\text{Estimated Daily Intake (EDI) } \mu\text{g/kg/day} = \frac{\text{Intake rate of edible seeds (g)} \times \text{AFs mean level } \mu\text{g/kg}}{\text{Average weight (kg)}}$$

A frequency questionnaire was used to estimate the consumption data of edible seeds used in rice, sweet dishes, and cold drinks to approximately 650 participants and asking them about the edible seeds utilized in the last 4 weeks. The questionnaire was completed by considering all aspects of the intake of seeds, their dietary supplements, and consistency. The exact quantity of edible seeds used in food products was evaluated from the survey. The male and female participants had an average weight of 71.5 and 50 kg, respectively.

2.7. Statistical Analysis

The data were analyzed as triplicate replicates, and the mean levels were given as standard deviations. The seven-point standard curve was constructed, a straight-line equation was obtained, and the coefficient of determination (R^2) was determined using linear regression. The significant differences in AFs levels in edible seeds from the summer and the winter seasons were determined using one-way ANOVA ($\alpha = 0.05$) using SPSS (IBM Inc., Armonk, NY, USA).

3. Results and Discussion

3.1. HPLC Method Validation

The HPLC parameters were evaluated in terms of the limit of detection (LOD) and the limit of quantification (LOQ). The LOD and LOQ of AFB₁ and AFB₂ were 0.05 and 0.15 µg/kg, and 0.08 and 0.24 µg/kg, respectively, for AFG₂ and AFB₂. The LOD and LOQ were measured as the signal-to-noise ratio of S/N (3), as shown in Table 1. The precision and accuracy were calculated using recovery analysis. The recovery examination was carried out by adding 4, 8, and 20 µg/kg levels of AFB₁ and AFG₁, and levels of 2, 4, and 12 µg/kg of AFB₂ and AFG₂ were added to negative samples of mixed edible seeds. The current procedure has revealed a good recovery, ranging from 75 to 110% with RSD (relative standard deviation) from 10 to 19%. A previous study [29] reported recoveries of AFs in dry fruits and edible seeds samples varied from 83–90% with RSD 8 to 19%, comparable to the results of the present study. The values of LOD and LOQ for AFB₁ and AFG₁ were 0.04 and 0.12 µg/L and 0.06 and 0.18 µg/L for AFG₂ and AFB₂, respectively. The natural occurrence of individual AFB₁, AFG₁, AFG₂, and AFB₂ in the pumpkin seed sample is shown in Figure 1.

Table 1. Analytical parameters for the determination of AFs and tocopherols.

Aflatoxins	Linearity $\mu\text{g/mL}$	LOD $\mu\text{g/kg}$	LOQ $\mu\text{g/kg}$	R^2	Precision (%RSD)	
					Reproducibility	Repeatability
AFB ₁	1–120	0.05	0.15	0.9981	17	15
AFB ₂	0.5–25	0.08	0.24	0.9947	14	16
AFG ₁	1–120	0.05	0.15	0.9972	12	17
AFG ₂	0.5–25	0.08	0.24	0.9972	10	16
α	0.5–60	0.04	0.12	0.9986	11	19
γ	0.1–30	0.07	0.21	0.9883	16	18
δ	0.1–30	0.07	0.21	0.9891	14	18

RSD: Relative Standard deviation; LOQ: Limit of Quantification; LOD: Limit of Detection. (α , γ , δ are the isomers of tocopherols).

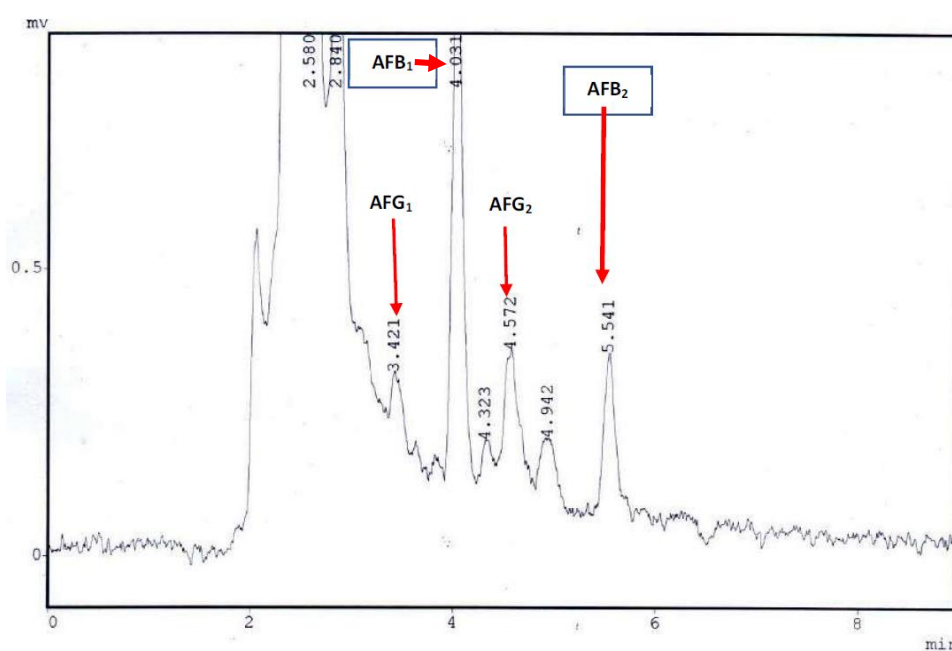


Figure 1. The natural occurrence of individual peaks corresponding to AFB₁, AFG₁, AFB₂, and AFG₂ in a pumpkin without shell seed sample.

3.2. Occurrence of AFs in Edible Seeds

The study was conducted for the examination of AFs in 414 samples of edible seeds (melon seeds, watermelon seeds, cantaloupe seeds, and pumpkin seeds) from the winter season and 365 samples of edible seeds from the summer season, and results are presented in Table 2. The maximum average of AFB₁ and total AFs (16.5 ± 2.45 and 20.9 ± 3.10 $\mu\text{g/kg}$) was found without shelled watermelon seed samples from the winter season. Furthermore, the maximum average of AFB₁ (14.4 ± 1.90 $\mu\text{g/kg}$) and total AFs (17.3 ± 1.50 $\mu\text{g/kg}$) was found without shell samples of pumpkin seed from the summer seasons. In shelled pumpkin seeds, the amount of AFs found in the summer season sample was 17.3 ± 1.50 $\mu\text{g/kg}$. In shelled watermelon seeds, the minimum level of AFB₁ and total AFs were 6.70 ± 1.90 and 8.4 ± 1.90 $\mu\text{g/kg}$, respectively, from the summer season. The findings showed that out of a total of 365 summer season experiments, 122 (33.4%) were aflatoxin-infected and 180 (43.5%) were aflatoxin poisoned out of 414 winter season experiments. The results have shown that 27.2% of samples of edible seeds having the levels of AFB₁ higher than EU permissible limit (≥ 5 $\mu\text{g/kg}$), and 12.2% of samples having total AFs levels greater than ≥ 20 $\mu\text{g/kg}$ from the summer season as presented in Figure 2. Furthermore, in 32.8% of samples levels of AFB₁ greater than 5 $\mu\text{g/kg}$ were

found and 11.5% of samples from the winter season had a total amount of AFs higher than 20 µg/kg, as shown in Figure 3.

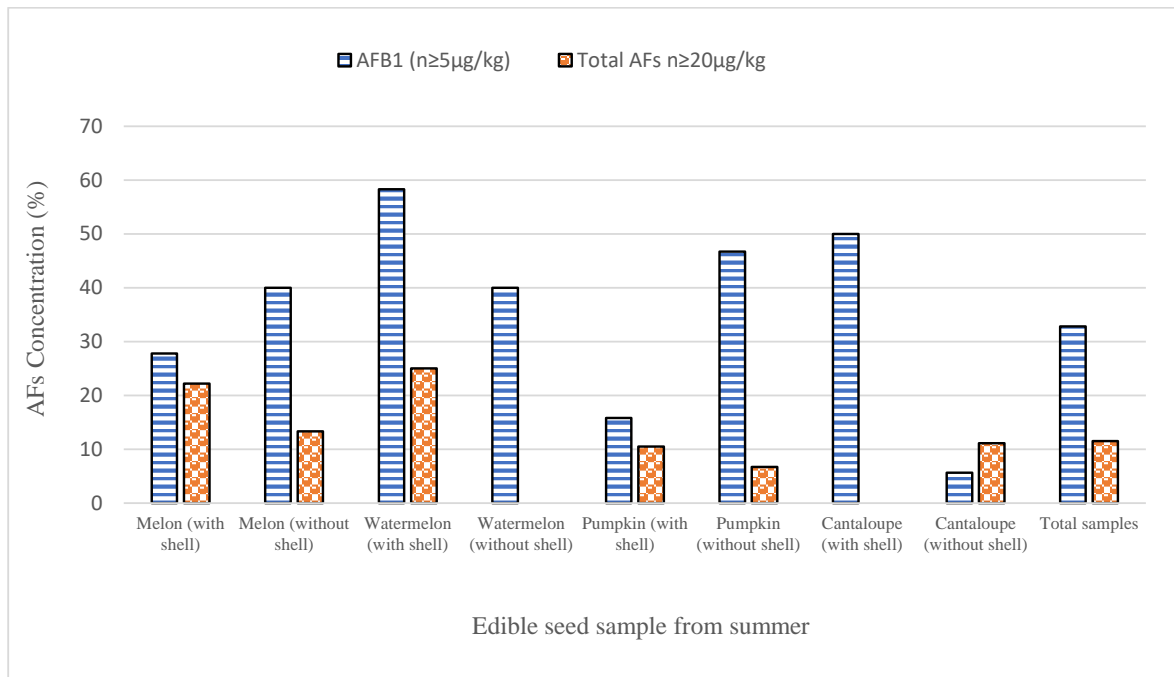


Figure 2. The number of AFB₁ and total AFs (µg/kg) samples higher than the recommended EU limits from the summer season.

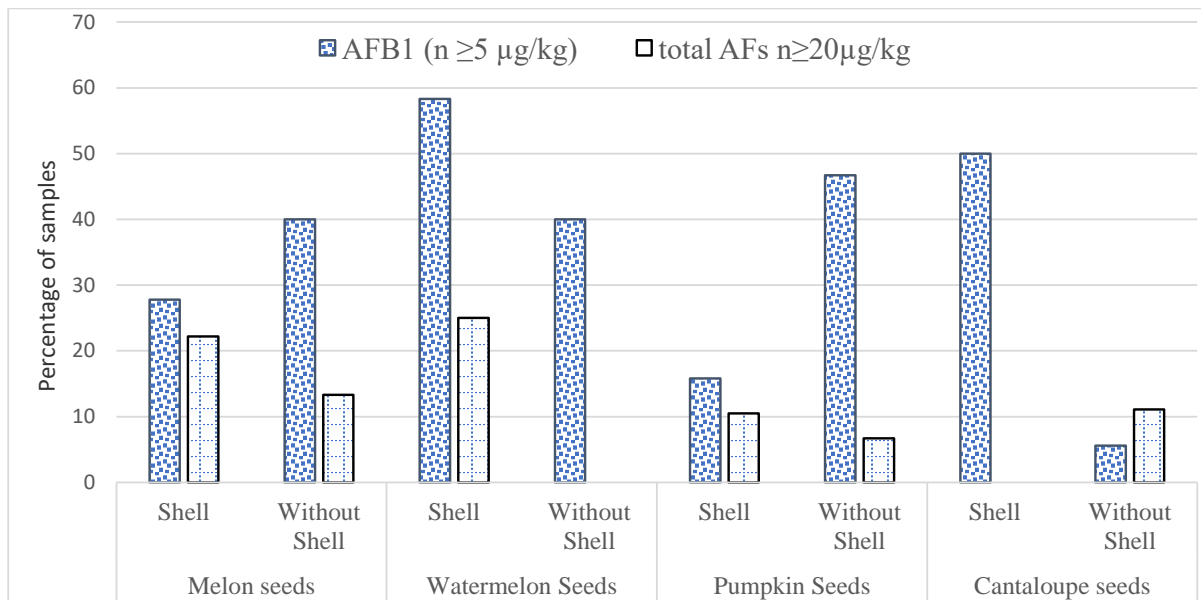


Figure 3. The number of AFB₁ and total AFs (µg/kg) samples higher than the recommended EU limits from the winter season.

Table 2. Occurrence of AFB₁ and total AFs ($\mu\text{g}/\text{kg}$) in edible seeds from summer and winter season from Punjab, Pakistan.

Seasons	Type	Melon Seeds		Watermelon Seeds		Pumpkin Seeds		Cantaloupe Seeds		Total
		Shelled	Without Shell	Shelled	Without Shell	Shelled	Without Shell	Shelled	Without Shell	
Winter Season	Total sample (<i>n</i>)	60	65	42	32	75	60	40	40	414
	Positive Sample <i>n</i> (%)	23 (38.33)	20 (30.76)	18 (42.85)	20 (62.5)	23 (30.66)	28 (46.66)	23 (57.5)	25 (62.5)	180 (43.4)
	AFB ₁ ($\mu\text{g}/\text{kg}$) \pm SD	10.5 \pm 2.10	12.6 \pm 2.50	8.90 \pm 2.80	16.5 \pm 2.45	12.9 \pm 2.60	12.6 \pm 3.40	7.9 \pm 2.60	11.8 \pm 3.20	
	Total AFs ($\mu\text{g}/\text{kg}$) \pm SD	13.5 \pm 3.40 *	15.9 \pm 3.60 *	11.1 \pm 2.10 **	20.9 \pm 3.10 **	13.5 \pm 2.90 **	18.5 \pm 2.90 **	13.5 \pm 3.10 *	16.5 \pm 3.50 *	
	Range ($\mu\text{g}/\text{kg}$)	0.05–25.40	0.05–35.5	0.05–20.5	0.05–35.5	0.05–28.5	0.05–38.9	0.05–50.5		
Summer Season	Total sample (<i>n</i>)	50	60	40	40	60	50	30	35	365
	Positive Sample (%)	18 (36)	15 (25)	12 (30)	15 (37.5)	19 (31.7)	15 (30)	10 (33.3)	18 (51.4)	122 (33.4)
	AFB ₁ ($\mu\text{g}/\text{kg}$) \pm SD	8.20 \pm 2.50	10.9 \pm 2.50	6.70 \pm 1.90	10.1 \pm 1.90	9.8 \pm 2.50	14.4 \pm 1.90	6.3 \pm 1.90	10.2 \pm 2.80	
	Total AFs ($\mu\text{g}/\text{kg}$) \pm SD	11.80 \pm 2.10 *	13.2 \pm 2.80 *	8.40 \pm 1.95 *	11.6 \pm 1.80 *	15.0 \pm 2.50 *	17.3 \pm 1.50 *	10.5 \pm 1.95 *	13.9 \pm 2.10 *	
	Range ($\mu\text{g}/\text{kg}$)	0.05–25.5	0.05–39.8	0.05–23.6	0.05–45.6	0.05–29.8	0.05–39.7	0.05–20.5	0.05–33.2	

* = The significant difference of variation in total AFs levels in edible seeds available shelled versus without shell ($\alpha = 0.05$). ** = The significant difference of variation in total AFs levels in edible seeds available shelled versus without shell ($\alpha = 0.01$).

In a previous study, Iqbal et al. [29] examined 320 samples of edible nuts (peanut, poppy seed, pistachio, almonds, cashew) and dry fruits (figs, plum, raisins, apricot, dates, watermelon seed, pomegranate seeds, and melon seeds) and observed that 128 (40%) samples were contaminated with AFB₁ and the incidence of total AFs was comparable to the results of the current findings. The samples had levels of total AFs greater than 4 µg/kg and 10 µg/kg in 34 and 25% of samples, and the elevated average amount of total AFs (7.30 ± 1.80 µg/kg), was comparatively low to the results of the present study. In another study, Masood et al. [30] investigated 307 samples of dried fruits and edible nuts from Pakistan and observed that 132 (43%) of the samples were found to be contaminated with AFB₁, and total AFs. The elevated average amount of total AFs i.e., 7.89 ± 0.99 µg/kg in peanuts without shell samples and the lowest average amount (2.45 ± 0.11 µg/kg) was observed in watermelon without shell samples. Huang et al. [35] have observed a very high percentage of samples from China to be contaminated with AFs, i.e., 93.9% of peanut butter, and documented an average amount ranging from 0.3 µg/kg to 95.9 µg/kg, a much higher range than the results of the present findings. Iqbal et al. [31] have analyzed 198 samples of peanut and peanut products from Pakistan and found that 61% of roasted peanut (shell samples), 68% of roasted peanut (without shell), 59%, of raw peanut (with shell), 55% of raw peanut (without shell), 50% of peanut butter, 42% of peanut cookies and 20% of peanut nemko were observed to be contaminated with AFs. The average concentration in raw peanut shell samples was 6.4 µg/kg, in roasted peanut with and without shell (10.4 and 12.3 µg/kg), in raw peanut without shell 9.6 µg/kg, peanut butter 2.4 µg/kg, peanut nimko (3.4 µg/kg), and in peanut cookies 4.6 µg/kg, much lower than the findings of the present study. A low percentage of AFs contamination and average amount have been documented by Kabak et al. for samples from Turkey [36]. The results demonstrated that among 300 samples of both dried figs and hazelnuts six (12%) of samples of hazelnut kernel (ranging from 0.09 to 11.3 µg/kg) and five (8.3%) samples of roasted hazelnut kernel (average 0.17 to 11.2 µg/kg) were found to be contaminated by AFs. Bankole et al. [37] analyzed 137 melon seed samples from Nigeria and documented a mean level of AFB₁ (14.8 µg/kg) in forest melon seed and 11.3 µg/kg in savanna melon seeds. In another study, Juan et al. [38] analyzed 100 samples of dried fruits and nuts from Morocco and documented a very high mean level of AFB₁ (2500 µg/kg) in walnut samples and an average level of 1430 µg/kg in pistachio samples, very high amounts compared to the results of the present findings. In the summer season, during the cultivation, the environmental conditions, especially the climatical variations, i.e., rainfall, broadly affects the growth of fungi in food products. Therefore, the weather and climatic conditions are considered important factors for AF production [39]. The fungal attack and levels of AFs in nuts and dried fruits may also vary during seasons. The months of July, August, and September are rainy periods with high rainfall levels and higher moisture and humidity levels [16,40]. The previous findings have shown that the most suitable temperature for the growth of various fungal species, particularly of *Aspergillus* species, ranges between 10.0 to 48.8 °C with 33.8 °C as an optimum temperature [38]. However, there was a significant difference in AFs levels in edible seeds from the shell and without shell samples ($p < 0.05$).

3.3. Dietary Intake Estimation

The estimation of dietary intake in edible seeds from the summer and the winter season in male and female individuals is presented in Table 3. The results have shown that maximum dietary intake of AFs was assessed to be 4.38 µg/day/kg body mass (BW) in males for pumpkin seeds without shell from the winter season and the maximum amount of 6.3 µg/day/kg BW was found in winter season pumpkin seeds for female individuals. The minimum dietary intake was documented without shell melon seeds, i.e., 1.04 µg/day/kg BW in the winter period in males and from the summer season 0.59 µg/day/kg BW in males. In earlier findings, Heshmati et al. [41] confirmed that the dietary intake in dates, dried mulberries, figs, and apricots was 0.12, 0.04, 0.04, and 0.06 ng/kg/bw/day, respectively. Williams et al. [42] have documented lower levels of dietary exposure compared the current findings, i.e., levels of AFs was 11.4 to 158.6 ng/kg/day in Swaziland, 3.5 to 14.8 ng/kg/day in Kenya, 38.6 to 183.7 ng/kg/day in Mozambique, 16.5 ng/kg/day in Transkei (South Africa), 4 to 115 ng/kg/day in

the Gambia, 11.7 to 2027 ng/kg/day from China, 6.5 to 53 ng/kg/day from Thailand, 2.7 ng/kg/day in the USA. Sugita-Konishi et al. [43], from Japan, have stated that the exposure for AFB₁ ranged from 0.908 ng/kg BW/day to 0.909 ng/kg BW/day in one to six year-old children and 0.288 ng/kg BW/day to 0.289 ng/kg BW/day in adults of age more than 20 years, much lower than the current results.

Table 3. Estimation of dietary intake for AFs in edible seeds in the local population from Punjab, Pakistan.

Seasons	Type	Melon Seeds		Watermelon Seeds		Pumpkin Seeds		Cantaloupe Seeds	
		Shelled	Without Shell	Shelled	Without Shell	Shelled	Without Shell	Shelled	Without Shell
	Consumption g/day	20	5	10	20	15	10	15	10
	AFs mean level (µg/kg)	13.5	14.9	11.1	13.5	20.9	18.5	13.5	14.5
Winter	Dietary Intake (male) µg/kg/day	3.78	1.04	1.55	3.78	2.59	4.38	2.83	2.03
	Dietary Intake (female) µg/kg/day	5.4	1.50	2.20	5.40	3.70	6.30	4.10	2.90
	AFs mean level µg/kg	11.8	12.20	8.40	11.60	17.30	15	10.50	11.90
Summer	Dietary Intake (male) µg/kg/day	1.65	0.85	0.59	1.62	1.21	2.10	1.47	0.83
	Dietary Intake (female) µg/kg/day	2.40	1.20	0.80	2.30	1.70	3.00	2.10	1.20

Average Weight female = 55, Average age = 27.4. Average Weight male = 71.5, Average age = 29.8.

3.4. The Vitamin E Levels in Edible Seeds

The variation of vitamin E levels in edible seeds from the winter and the summer season is presented in Table 4.

Table 4. Detection of vitamin E content in edible seeds from Punjab, Pakistan.

Seasons	Seeds Type	Samples	α Tocopherol (mg/100 g)	γ Tocopherol (mg/100 g)	δ Tocopherol (mg/100 g)	Total Tocopherol Content (mg/100 g)
Winter Season	Melon seeds	125	11.5 ± 2.70	4.5 ± 2.30	3.5 ± 1.90	19.5 ± 4.90 *
	Watermelon seeds	74	0.93 ± 1.40	0.01 ± 4.15	2.91 ± 1.05	3.85 ± 3.60 ^{NS}
	Pumpkin seeds	135	12.1 ± 2.80	2.0 ± 2.50	8.1 ± 2.10	22.2 ± 7.70 *
	Cantaloupe seeds	80	8.07 ± 1.30	4.03 ± 1.15	3.02 ± 1.10	15.12 ± 4.65 *
Summer Season	Melon seeds	110	9.5 ± 3.75	2.5 ± 2.80	2.5 ± 2.94	14.5 ± 5.50 *
	Watermelon seeds	80	1.04 ± 2.45	0.01 ± 1.15	1.91 ± 1.45	2.96 ± 5.60 ^{NS}
	Pumpkin seeds	110	8.1 ± 3.85	1.50 ± 2.10	4.15 ± 3.15	13.75 ± 6.50 *
	Cantaloupe seeds	65	4.05 ± 3.35	2.04 ± 3.10	2.02 ± 3.15	8.11 ± 3.40 *

* = The variation in total levels of tocopherols shows a significant difference in winter than summers seasons samples ($\alpha = 0.05$). ^{NS} = The variation in total levels of tocopherols shows non-significant difference in winter compared to summers seasons samples ($\alpha = 0.05$).

The maximum average amount of total tocopherol content was found in melon seeds, i.e., 19.5 ± 4.90 mg/100 g, and the lowest total tocopherol content was observed in watermelon seeds 3.85 ± 3.60 mg/100 g from the winter season. However, the highest level of total tocopherol 14.5 ± 5.50 mg/100 g was found in melon seeds, and the most moderate mean level of 2.96 ± 5.60 mg/100 g was found in watermelon seeds from the summer season. The findings reveal the significant differences in vitamin E levels in samples from the winter and summer seasons ($\alpha = 0.05$), except for watermelon seeds, which show non-significant differences ($\alpha = 0.05$). The individual and total levels of vitamin E content in winter and summer seasons are presented in Figure 4.

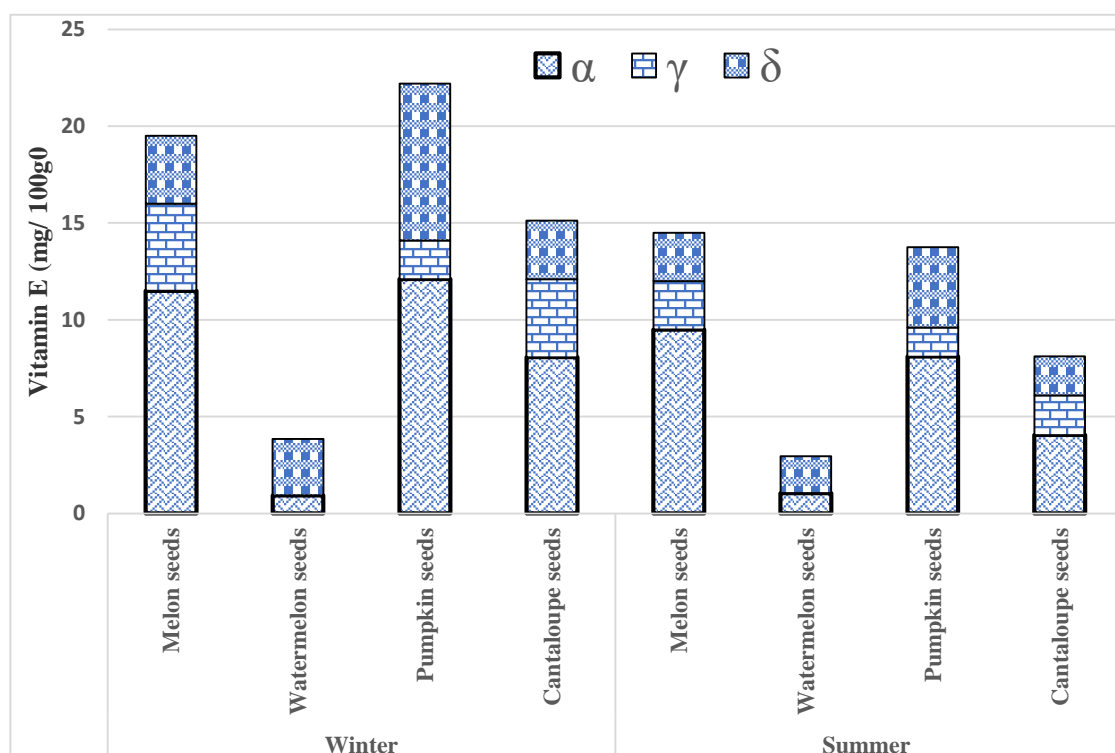


Figure 4. The variation of individual and total tocopherol in edible seed samples from winter and summer seasons.

The study has observed a negative correlation (Pearson correlation -0.370 , and shown a significant difference between vitamin E and total AFs levels at $\alpha = 0.01$) between AFs amount in different edible seeds and vitamin E content from winter and summer seasons, as shown in Figure 5.

In a previous study Iqbal et al. [19] have shown significant differences ($p < 0.05$) in vitamin E contents in different rice varieties, and a negative correlation ($r = -0.62$) was found between AFs concentration and vitamin E levels, in agreement with the present findings. Previous studies have observed that selenium and retinol's antioxidant properties, ascorbic acid, and tocopherol not only safeguard the membrane from the harmful effect of mycotoxins, but induce or boost the liver function to detoxify mycotoxin levels [44]. Furthermore, the extracts from certain medicinal herbs and plants could possibly defend from ochratoxin A, aflatoxin B1, and fumonisin B1 toxicity [45–47]. More comprehensive work to establish a relationship between AFs in edible seeds and variation in vitamin E content is recommended.

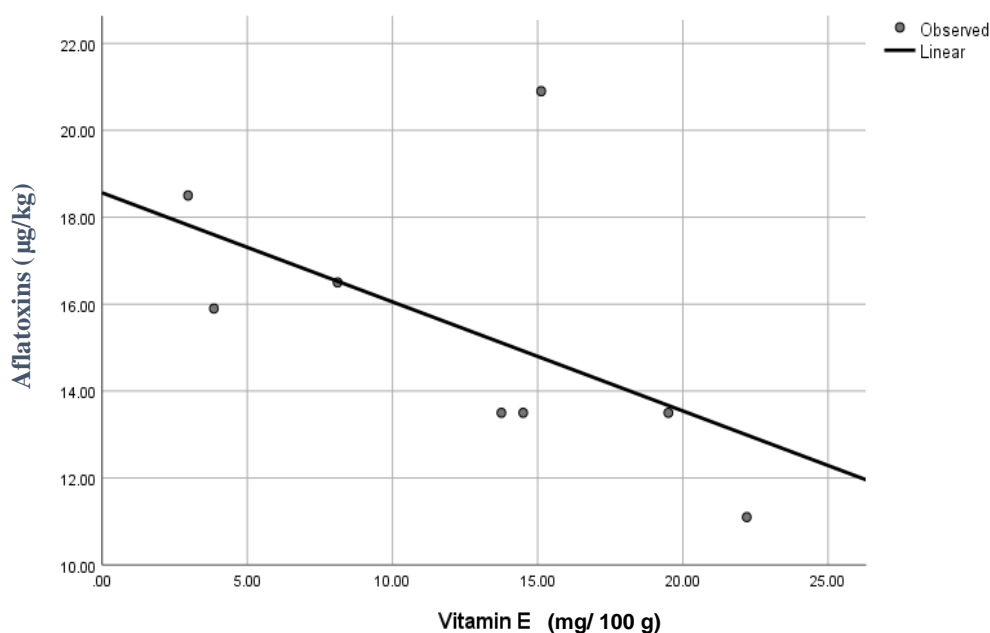


Figure 5. The correlation between vitamin E and total AFs concentrations.

4. Conclusions

The average amount of total AFs in edible seeds from the winter and summer seasons was considerably high compared to our previous studies. Furthermore, no significant difference in AFs levels was found in winter versus the summer season. However, there exists a significant difference in AFs variation in edible seeds samples consumed with shells versus without shell samples. The highest dietary intake of 6.30 µg/kg/day was calculated in female individuals from pumpkin seeds samples from the winter season. The highest vitamin E levels 22.2 ± 7.70 mg/100 g were found in pumpkin seeds from winter samples. There exists a negative correlation between AFs levels and vitamin E contents in edible seeds. The present research results should be informative for farmers, traders and consumers regarding the health consequences correlated with these toxins. We have purchased these edible seeds available in the market with shells or without shells, but in the future, we would be more interested in collecting samples during the field with fruits and then studying the pre-harvest content of fungi or aflatoxins.

Author Contributions: Conceptualization, writing—original draft preparation, supervision, visualization, project administration, & investigation, S.Z.I.; methodology, software, and formal analysis, M.M.S. and N.N.; validation, M.R.A.; resources, data curation, N.B.A. and S.U.; writing—review and editing, A.F.A.R. All authors have read and agreed to the published version of the manuscript.

Funding: The authors recognize the financial assistance of Higher Education Commission, Islamabad, (NRPU-7754). The APC was funded by Universiti Putra Malaysia.

Acknowledgments: The analytical facilities offered by NIAB, Faisalabad are highly valued.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Sugizaki, C.S.A.; Naves, M.M.V. Potential prebiotic properties of nuts and edible seeds and their relationship to obesity. *Nutrients* **2018**, *10*, 1645. [[CrossRef](#)] [[PubMed](#)]
2. Mora-Escobedo, R.; Berrios, J.D.J.; Gutiérrez-Lopez, G.F. Seeds as functional foods and nutraceuticals. *New Front. Food Sci.* **2014**, *91*, 220–225.
3. Patel, S.; Rauf, A. Edible seeds from Cucurbitaceae family as potential functional foods: Immense promises, few concerns. *Biomed. Pharmacother.* **2017**, *91*, 330–337. [[CrossRef](#)] [[PubMed](#)]

4. Grancieri, M.; Martino, H.S.D.; De Mejia, E.G. Chia seed (*Salvia hispanica* L.) as a source of proteins and bioactive peptides with health benefits: A review. *Compr. Rev. Food Sci. Food Saf.* **2019**, *18*, 480–499. [[CrossRef](#)]
5. Hernández-Mesa, M.; García-Campaña, A.M. Determination of sulfonylurea pesticide residues in edible seeds used as nutraceuticals by QuEChERS in combination with ultra-high-performance liquid chromatography-tandem mass spectrometry. *J. Chromatogr. A* **2020**, *1617*, 460831. [[CrossRef](#)] [[PubMed](#)]
6. Bardaa, S.; Ben Halima, N.; Aloui, F.; Ben Mansour, R.; Jabeur, H.; Bouaziz, M.; Sahnoun, Z. Oil from pumpkin (*Cucurbita pepo* L.) seeds: Evaluation of its functional properties on wound healing in rats. *Lipids Health Dis.* **2016**, *15*, 1–12. [[CrossRef](#)]
7. Set, E.; Erkmen, O. The aflatoxin contamination of ground red pepper and pistachio nuts sold in Turkey. *Food Chem. Toxicol.* **2010**, *48*, 2532–2537. [[CrossRef](#)]
8. Bhat, R.; Rai, R.V.; Karim, A.A. Mycotoxins in food and feed: Present status and future concerns. *Compr. Rev. Food Sci. Food Saf.* **2010**, *9*, 57–81. [[CrossRef](#)]
9. Abbas, H.K.; Shier, W.T.; Plasencia, J.; Weaver, M.; Bellaloui, N.; Kotowicz, J.K.; Butler, A.M.; Accinelli, C.; De La Torre-Hernandez, M.E.; Zablutowicz, R.M. Mycotoxin contamination in corn smut (*Ustilago maydis*) galls in the field and in the commercial food products. *Food Control.* **2017**, *71*, 57–63. [[CrossRef](#)]
10. Alassane-Kpembi, I.; Schatzmayr, G.; Taranu, I.; Marin, D.; Puel, O.; Oswald, I.P. Mycotoxins co-contamination: Methodological aspects and biological relevance of combined toxicity studies. *Crit. Rev. Food Sci. Nutr.* **2017**, *57*, 3489–3507. [[CrossRef](#)]
11. Alberts, J.; Lilly, M.; Rheeder, J.; Burger, H.-M.; Shephard, G.; Gelderblom, W. Technological and community-based methods to reduce mycotoxin exposure. *Food Control.* **2017**, *73*, 101–109. [[CrossRef](#)]
12. Eskola, M.; Kos, G.; Elliott, C.T.; Hajšlová, J.; Mayar, S.; Krska, R. Worldwide contamination of food-crops with mycotoxins: Validity of the widely cited ‘FAO estimate’ of 25%. *Crit. Rev. Food Sci. Nutr.* **2020**, *60*, 2773–2789. [[CrossRef](#)]
13. Pleadin, J.; Vahčić, N.; Perši, N.; Ševelj, D.; Markov, K.; Frece, J. Fusarium mycotoxins’ occurrence in cereals harvested from Croatian fields. *Food Control.* **2013**, *32*, 49–54. [[CrossRef](#)]
14. Zinedine, A.; Fernández-Franzón, M.; Mañes, J.; Manyes, L. Multi-mycotoxin contamination of couscous semolina commercialized in Morocco. *Food Chem.* **2017**, *214*, 440–446. [[CrossRef](#)] [[PubMed](#)]
15. Morris, J.G., Jr.; Potter, M. *Foodborne Infections and Intoxications*; Academic Press: Cambridge, MA, USA, 2013.
16. Asghar, M.A.; Ahmed, A.; Zahir, E.; Iqbal, J.; Walker, G. Incidence of aflatoxins contamination in dry fruits and edible nuts collected from Pakistan. *Food Control.* **2017**, *78*, 169–175. [[CrossRef](#)]
17. Ismail, A.; Gonçalves, B.L.; De Neeff, D.V.; Ponzilacqua, B.; Coppa, C.F.; Hintzsche, H.; Sajid, M.; Cruz, A.G.; Corassin, C.H.; Oliveira, C.A. Aflatoxin in foodstuffs: Occurrence and recent advances in decontamination. *Food Res. Int.* **2018**, *113*, 74–85. [[CrossRef](#)]
18. De Roma, A.; Rossini, C.; Ritieni, A.; Gallo, P.; Esposito, M. A survey on the Aflatoxin M1 occurrence and seasonal variation in buffalo and cow milk from Southern Italy. *Food Control.* **2017**, *81*, 30–33. [[CrossRef](#)]
19. Iqbal, S.Z.; Mustafa, H.G.; Asi, M.R.; Selamat, J. Variation in vitamin E level and aflatoxins contamination in different rice varieties. *J. Cereal Sci.* **2014**, *60*, 352–355. [[CrossRef](#)]
20. Ruadrew, S.; Craft, J.; Aidoo, K. Occurrence of toxigenic *Aspergillus* spp. and aflatoxins in selected food commodities of Asian origin sourced in the West of Scotland. *Food Chem. Toxicol.* **2013**, *55*, 653–658. [[CrossRef](#)]
21. Blankson, G.; Mill-Robertson, F. Aflatoxin contamination and exposure in processed cereal-based complementary foods for infants and young children in greater Accra, Ghana. *Food Control.* **2016**, *64*, 212–217. [[CrossRef](#)]
22. World Health Organization & International Agency for Research on Cancer. Some naturally occurring substances: Food items and constituents, heterocyclic aromatic amines and mycotoxins. In *IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans*; IARC Publication: Lyon, France, 1993.
23. UN Food and agriculture organization of the United Nations (FAO). *Yearbook of the United Nations 2004*; United Nations Publications: New York, NY, USA, 2004; Volume 56, pp. 1470–1471.
24. European Commission (EC). Commission Regulation (EC) No 165/2010 of 26 February 2010, amending Regulation (EC) No 1881/2006 setting maximum levels for certain contaminants in foodstuffs as regards aflatoxins. *Off. J. Eur. Union* **2010**, *50*, 8–12.
25. Nesci, A.; Gsponer, N.; Etcheverry, M. Natural maize phenolic acids for control of aflatoxigenic fungi on maize. *J. Food Sci.* **2007**, *72*, M180–M185. [[CrossRef](#)] [[PubMed](#)]

26. Samapundo, S.; De Meulenaer, B.; Osei-Nimoh, D.; Lamboni, Y.; Debevere, J.; Devlieghere, F. Can phenolic compounds be used for the protection of corn from fungal invasion and mycotoxin contamination during storage? *Food Microbiol.* **2007**, *24*, 465–473. [CrossRef] [PubMed]
27. Sýs, M.; Švecová, B.; Švancara, I.; Metelka, R. Determination of vitamin E in margarines and edible oils using square wave anodic stripping voltammetry with a glassy carbon paste electrode. *Food Chem.* **2017**, *229*, 621–627. [CrossRef] [PubMed]
28. Iqbal, S.Z.; Mumtaz, A.; Mahmood, Z.; Waqas, M.; Ghaffar, A.; Ismail, A.; Pervaiz, W. Assessment of aflatoxins and ochratoxin a in chili sauce samples and estimation of dietary intake. *Food Control* **2021**, *121*, 107621. [CrossRef]
29. Iqbal, S.Z.; Mehmood, Z.; Asi, M.R.; Shahid, M.; Sehar, M.; Malik, N. Co-occurrence of aflatoxins and ochratoxin A in nuts, dry fruits, and nuty products. *J. Food Saf.* **2018**, *38*, e12462. [CrossRef]
30. Masood, M.; Iqbal, S.Z.; Asi, M.R.; Malik, N. Natural occurrence of aflatoxins in dry fruits and edible nuts. *Food Control.* **2015**, *55*, 62–65. [CrossRef]
31. Iqbal, S.Z.; Asi, M.R.; Zuber, M.; Akram, N.; Batool, N. Aflatoxins contamination in peanut and peanut products commercially available in retail markets of Punjab, Pakistan. *Food Control.* **2013**, *32*, 83–86. [CrossRef]
32. Bankole, S.A.; Adenusi, A.A.; Lawal, O.; Adesanya, O. Occurrence of aflatoxin B1 in food products derivable from ‘egusi’ melon seeds consumed in southwestern Nigeria. *Food Control.* **2010**, *21*, 974–976. [CrossRef]
33. Gliszczynska-Swigło, A.; Sikorska, E. Simple reversed-phase liquid chromatography method for determination of tocopherols in edible plant oils. *J. Chromatogr. A.* **2004**, *1048*, 195–198. [CrossRef]
34. McGuire, S. *WHO, World Food Programme, and International Fund for Agricultural Development. 2012; The State of Food Insecurity in the World 2012: Economic Growth is Necessary but not Sufficient to Accelerate the Reduction of Hunger and Malnutrition*; FAO: Rome, Italy, 2014.
35. Huang, B.; Han, Z.; Cai, Z.; Wu, Y.; Ren, Y. Simultaneous determination of aflatoxins B1, B2, G1, G2, M1 and M2 in peanuts and their derivative products by ultra-high-performance liquid chromatography–tandem mass spectrometry. *Anal. Chim. Acta* **2010**, *662*, 62–68. [CrossRef]
36. Kabak, B. Aflatoxins in hazelnuts and dried figs: Occurrence and exposure assessment. *Food Chem.* **2016**, *211*, 8–16. [CrossRef]
37. Bankole, S.A.; Ogunsanwo, B.; Osho, A.; Adewuyi, G. Fungal contamination and aflatoxin B1 of ‘egusi’ melon seeds in Nigeria. *Food Control.* **2006**, *17*, 814–818. [CrossRef]
38. Juan, C.; Zinedine, A.; Moltó, J.; Idrissi, L.; Mañes, J. Aflatoxins levels in dried fruits and nuts from Rabat-Salé area, Morocco. *Food Control.* **2008**, *19*, 849–853. [CrossRef]
39. Pakistan Meteorological Department. Pakistan Meteorological Department Pakistan Government. 2016. Available online: <https://www.pmd.gov.pk/en/> (accessed on 5 November 2020).
40. Pitt, J.I.; Hocking, A.D. *Fungi and Food Spoilage*; Springer: New York, NY, USA, 2009.
41. Heshmati, A.; Zohrevand, T.; Khaneghah, A.M.; Nejad, A.S.M.; Sant’Ana, A.S. Co-occurrence of aflatoxins and ochratoxin A in dried fruits in Iran: Dietary exposure risk assessment. *Food Chem. Toxicol.* **2017**, *106*, 202–208. [CrossRef] [PubMed]
42. Williams, J.H.; Phillips, T.D.; Jolly, P.E.; Stiles, J.K.; Jolly, C.M.; Aggarwal, D. Human aflatoxicosis in developing countries: A review of toxicology, exposure, potential health consequences, and interventions. *Am. J. Clin. Nutr.* **2004**, *80*, 1106–1122. [CrossRef] [PubMed]
43. Sugita-Konishi, Y.; Sato, T.; Saito, S.; Nakajima, M.; Tabata, S.; Tanaka, T.; Norizuki, H.; Itoh, Y.; Kai, S.; Sugiyama, K.; et al. Exposure to aflatoxins in Japan: Risk assessment for aflatoxin B1. *Food Addit. Contam. Part. A* **2010**, *27*, 365–372. [CrossRef]
44. Da Silva, E.; Bracarense, A.P.F.; Oswald, I. Mycotoxins and oxidative stress: Where are we? *World Mycotoxin J.* **2018**, *11*, 113–134. [CrossRef]
45. Mohajeri, M.; Behnam, B.; Cicero, A.F.G.; Sahebkar, A. Protective effects of curcumin against aflatoxicosis: A comprehensive review. *J. Cell. Physiol.* **2017**, *233*, 3552–3577. [CrossRef]

46. Tabeshpour, J.; Mehri, S.; Behbahani, F.S.; Hosseinzadeh, H. Protective effects of *Vitis vinifera* (grapes) and one of its biologically active constituents, resveratrol, against natural and chemical toxicities: A comprehensive review. *Phytother. Res.* **2018**, *32*, 2164–2190. [[CrossRef](#)]
47. Hedayati, N.; Naeini, M.B.; Nezami, A.; Hosseinzadeh, H.; Wallace Hayes, A.; Hosseini, S.; Imenshahidi, M.; Karimi, G. Protective effect of lycopene against chemical and natural toxins: A review: Lycopene against chemical and natural toxins. *BioFactors* **2019**, *45*, 5–23. [[CrossRef](#)] [[PubMed](#)]

Publisher’s Note: MDPI stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.



© 2020 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<http://creativecommons.org/licenses/by/4.0/>).