Evaluation of Aflatoxin Contamination in Raw and Roasted Nuts in Consumed Kerman and Effect of Roasting, Packaging and Storage Conditions

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Abstract: Background: Aflatoxins (AFs) are a group of mycotoxins and carcinogenic secondary fungal metabolites and have been detected in various food commodities including nuts. The aim of the present study was to investigate the presence of AFs in nuts available in Kerman, in center of Iran. Material and methods: In this study, 85 samples of nuts (raw pistachio, walnut, almond, peanut, roasted pistachio and roasted peanut) were collected from different areas of local markets in Kerman, Iran, analyzed for aflatoxin B1 (AFB1), aflatoxin B2 (AFB2), aflatoxin G1 (AFG1) and aflatoxin G2 (AFG2) using by high-performance liquid chromatography with fluorescence detection after immune-affinity column clean up with recoveries ranging from 77 to 99. Results: In this regard, result showed that AFB1 level in 11 samples (12.5%) was above the maximum tolerated level (MTL) EU limit. The highest contamination was found in the samples of raw pistachios. Aflatox in for all samples of walnuts were LOQ range. The aflatoxin G1 and G2 for all samples was in the range of LOQ. Mean aflatoxin contamination in the roasted samples (16.53 µg/kg) was significantly higher than the raw nuts (7.25 µg/kg) (P-value<0.001). After measuring the moisture content of the samples and Statistical analysis, there was a significant correlation between the aflatoxin and the amount of moisture in the samples (P- value<0.001). Conclusion: According to the high consumption of nuts and harmful effects of aflatoxin, should be considered as a health hazard.

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Introduction

Aflatoxins (AFs) are a group of toxic and carcinogenic polyketide secondary metabolites, which areproduced by strains of Aspergillus flavus, Aspergillus parasiticus, Aspergillus nomius and Aspergillus pseudotamarii on growing on a variety of food products, such as nuts, grains, dried fruits, and spices.^{[1], [2], [3]}

Although 20 aflatoxins have been identified, only 4 of them, that is the aflatoxins B1, B2, G1 and G2 (AFB1, AFB2, AFG1 and AFG2), occur naturally and are significant contaminants of a wide variety of foods and feeds.^{[4], [5]} AFB1 is the most potent carcinogenic substance naturally produced by Aspergillus species. Indeed, AFB1 is classified by the International Agency of Research on Cancer as Group1 carcinogen.^[6] There are rules for AFs limitation in nuts in many countries, European Communities set the maximum level for AFB₁ in a range of commodities for human consumption at 2ng/g.^[7] Recently, European Communities increased the maximum level in almonds, pistachios to 8ng/g and that for AFT from 4 to 10ng/g.^[8]

The Institute of Standards and Industrial Research of Iran^[9] put national legal limit to $15\mu g/kg$.^[9] Natural occurrence of AF in pistachio nuts has been studied in various countries, pistachio nuts are among the commodities with the highest risk of AF contamination.^[10] According to a report from Mexico, 2.2% of pistachio nut samples analyzed contained AF higher than 20ng/g.^[11]

In another study in Saudi Arabia, the concentration of AF in peanuts was $28\mu g/kg$. In some countries like Iran, processing of nuts (roasting) has shown to increase the AF levels of nuts.^[12] Nuts AF is not influenced by temperature and remains active even in 160°C.^[13]

Generally, tropical conditions such as high temperatures and moisture, monsoons, unseasonal rains during harvest, and flash flood lead to fungal proliferation and production of aflatoxins. Poor harvesting practices, improper storage, and marketing can also contribute to fungal growth and increase the risk of aflatoxin contamination.^[14]

Nuts have an important role among Iranian's food habits. Natural contamination of nuts with aflatoxin is unavoidable and causes a special challenge for nuts safety and quality. The aim of the present study was to investigate the presence of AFs and the sub-types of AFs in the various nuts available in Kerman (center of Iran) market.

Materials and methods Sampling

A total of 85 different types of nuts (raw pistachio, roasted pistachio, walnut, almond, peanut and roasted peanut) in both bulk and packaged formats were purchased according to random sampling from private markets in from different parts of Kerman city. The samples were placed in sterile plastic container and kept in a cool place until analyzed.

Sample preparation:

Water slurry of nut samples was prepared to minimize the sub sampling errors in aflatoxin analysis. Therefore, 1.5 L water was added to each 1 kg sub-sample of pistachio nut, followed by mixing and grinding the mixture by using a slurry machine for 15 minutes. When ready, 50 g of slurry was taken as the test portion for analysis.

Chemicals and reagents

Aflatoxin B1, B2, G1 and G2 standards were procured from Sigma (MO, U.S.A). Methanol, acetonitrile (Caledon laboratories Ltd, Can a1d4a2) were high-performance and water liquid chromatography (HPLC) grade. Sodium chloride, potassium bromide, nitric acid (Merck, Darmstadt, Germany) and phosphate-buffered saline [pH 7.4; 0.20 g KCl, 0.20 g K HPO4, 1.16g anhydrous Na2HPO4 (or 2.92 g Na2HPO4, 12H2O) and 8.0 g NaCl dissolved in 900 ml water and pH adjusted to 7.4 with 0.1 M HCl or 0.1 MNaOH and diluted to 1 L with water] were used in the present research.

Standard solutions

After preparation of standard solutions of each aflatoxin, the concentration was determined by using an Flourescense Detector (FLD) (Waters, 474, USA) through AOAC Official method No. 971.22 (AOAC, 2006, chap. 49.2.03). These standards were used to prepare mixed standards for HPLC analysis. The working standard solution was prepared by diluting mixed standards, Secondary stock standard 1000 ng/ml (AFB1, AFG1= 1000ng/g; AFB2, AFG2=200ng/g), with methanol and water.

Apparatus

Liquid chromatography (LC) analysis was performed using are verse-phase HPLC system (Waters, 2695, USA) equipped with a Gilson-Workstation (GX-271 Aspec Gilson, USA) fluorecence detector (Waters 474. USA). The Capital HPLC LC column was C18, $15cm \times 4.6 mm$, 5 µm. Aflatoxin immuno-affinity columns (IAC) were purchased from R-Biopharm (Darmstadt, Germany).

Chromatographic conditions

Reversed-phase LC determination of aflatoxins was performed using the post-column bromination with KobraCell (R-Biopharm Darmstadt, Germany) with a flow rate of 1 ml/min and fluorescence detectionat excitation wavelength 365nm and emission wavelength 435 nm. Retention times for AFG AFG1, AFB2, and AFB1 were 6.067, 7.122, 8.101, and 9.677 min, respectively. The isocratic mobile phase was water-acetonitrile-methanol solution and a ratioof 60:20:30(v/v/v), containing 120 mg/L KBr and 350 µl HNO3 4M.

Extraction and clean up

The samples were analyzed for aflatoxins content based on the AOAC Official Method No. $999.07{:}2000$ (AOAC, 2006, chap. 49.2.29) with minor modifications. $^{\left[1\right]}$ Pistachio nut slurries were extracted with methanol/hexane (120 ml /100 ml) after which the extract was filtered through What man Grade 1 Qualitative Filter Paper. After filtration, 20ml of extract was diluted with 130ml deionized water. For clean up the diluted extract, R-Biopharmimmuno-affinity columns were used. First, 10ml phosphate buffer saline was passed through the column by workstation clean needle. Then, 70 ml of the diluted was passed through the immune affinity column at flow rate of ca. 1 drop/s. The column was washed with 15 ml water and dried by applying little vacuum. Finally, aflatoxin was eluted with 1.5 ml methanol that was applied on the column which passed by gravity. The eluate was diluted with 1.5 ml deionized water, after which100µl was injected into HPLC.

Calibration curve

A Calibration curve was prepared by using the working standard solutions. The tertiary stock standard was used to prepare the working standard solutions by pipetting appropriate volumes of a mixed standard solution (1000ppm for B_1 and G_1 and 200ppm for B₂ and G₂) into a 4ml vial and take to 2ml volume in the way that the final concentration of solution be 100ppb for B_1 and G_1 and 20ppb for B_2 and G₂, respectively. The working standard solution was prepared by pippeting appropriate volumes of prepared stock solution to 4ml vial and take to 2ml volume in the way that final concentration of solution be 7.2, 5., 3.6, 2.8, 2, 1.2, 0.4 ppb for B_1 and G_1 respectively. A seven point calibration curve was built for each type of aflatoxin. The calibration curve was constructed before the analysis to check the pilot for linearity ($R^2 = 0.999$) and was used for quantification of aflatoxin. If the content of toxins in the sample was outside the calibration range, a more appropriate calibration curve was prepared, or the injection solution for LC analysis was diluted to an aflatoxin concentration appropriate for the established calibration curve.

Quality assurance:

For evaluation of the reliability of results, in addition to using validated methods, internal and external quality control experiments were performed. Regarding internal quality control, the accuracy and precision of the methods were verified. For this purpose, recoveries of AFB1, B2, G1 and G2 were recorded by analyzing a blank pistachio nut sample spiked at 4ng/g for AFB1 and AFG1, and 1ng/g for AFB2 and AFG2. According to the recovery values, the aflatoxin levels were corrected for recoveries.

Statistical analysis

Data of this study were entered to SPSS version 16 for statistical analysis. The qualitative data were analyzed including determination of correlation between the nominal variables by k-square. The quantitative data when normality was confirmed were analyzed were compared by ANOVA, otherwise the parallel non-parametric test was applied In all measurements P-value less than 0.5 was considered statistically significant.

Results

The collected 85 samples of nuts included 20 Raw Pistachio, 14, 7, 17, 14 and 13, samples of Walnut, Roasted Pistachio, Almond, Peanut, Roasted peanut, respectively. Aflatoxin levels in the nuts were analyzed by HPLC. In the current study the amounts of AFs (B1, B2,G1, G2, and Total) of six types of Nuts which are commonly consumed by people in Kerman were determined in the scale of μ g/kg.

Our results showed that 26.47% of the samples nuts were aflatoxin positive, the total average concentration of aflatoxin in all studied nuts was $34.325\mu g/kg.20$. Among total 58% of nuts samples contained aflatoxins higher than EU limit (4 $\mu g/kg$), and aflatoxin amounts in 17.64% of studied samples were higher than legal limit of national standard of Iran (15 $\mu g/kg$).

The obtained results also showed that the mean of total AF levels in the roasted samples $(13.62\pm 22.96\mu g/kg)$ that was more than raw nuts $(6.53\pm 18.77\mu g/kg)$ but the highest levels of AFs were found in Raw Pistachio sample.

Types of nut	Number of sample	Range	Mean	Std. Deviation
Raw Pistachio	20	85.24	13.5943	25.83405
Roasted Pistachio	7	38.27	9.9029	14.49489
Almond	17	85.33	7.5247	21.32710
Walnut	14	Nd ^b	Nd	Nd
Peanut	14	16.20	1.1957	4.31963
Roasted peanut	13	86.59	14.5854	26.31774

Table 1. Presence of Total aflatoxinsin^a different nuts by HPLC analysis

a Total aflatoxin was represented by the summation of aflatoxin B1, B2, G1 and G2 levels b Not detected (below the quantification limit); the mean of individual aflatoxin was calculated by assuming that the level of each aflatoxin in samples below the detection limit was equal to zero

Table 2. Presence of Total aflatoxins in Roasted nuts and Raw nuts by HPLC analysis

Types of nut	Ν	Mean	Std. Deviation	Std. Error Mean
Roasted nuts	19	13.6279	22.96788	5.26919
Raw nuts	66	6.5370	18.77451	2.31098

The mean of AFs (B1, B2, G1, G2, and Total) levels in the nuts show that in Table 3. The mean of AFB1 was more than legal limit of national standard of Iran ($5\mu g/kg$).

Type of	Number of sample	Range	Mean	Std. Deviation					
Total Aflatoxin	85	86.59	8.1067	19.87147					
AFB ₁	85	73.98	6.4415	16.21098					
AFB ₂	85	16.50	1.6205	3.65549					
AFG ₁	85	Nd ^a	Nd	Nd					
AFG ₂	85	Nd	Nd	Nd					

Table 3.	The mean	of AFs (Έ1.	B2 .	G1.	G2	and	Total)) leve	ls in	the	nuts
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a Not detected (below the quantification limit); the mean of individual aflatoxin was calculated by assuming that the level of each aflatoxin in samples below the detection limit was equal to zero

The moisture content, peroxide index, organoleptic properties, the presence of foreign matter and pests was measured. Table 3 shows the percentages of moisture content in different nuts.

Types of nut	Number of sample	Mean	Std. Dev	Min	Max
Raw Pistachio	20	6.07	1.66	4.3	8.3
Roasted Pistachio	7	5.2	2.47	2.2	5.2
Almond	17	3.92	0.87	3	5.5
Walnut	14	2.75	0.320	2.3	3.2
Peanut	14	4.77	0.57	4	5.3
Roasted peanut	13	2.75	1.34	1.7	4.5

After measuring the moisture content of the samples and Statistical analysis, there was a significant direct correlation between the aflatoxin and the amount of moisture in the samples (P-value<0.001).

Table 5. I decentages of contamination in unreferring	Table 5.	Percentages	of Cont	tamination	in	different nuts
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Test	aflatoxin	moisture content	peroxide index	organoleptic properties	presence of foreign matter	pests
Percentage of Contamination	26.47	23.52	14.71	20.58	26.47	5.88

High moisture content, pests and also peroxide level had a significant effect on the organoleptic properties of the nuts. Between Peroxide and aflatoxin there was no significant effect. As well between undesirable organoleptic properties and aflatoxin was no significant relationship.

Finally, the presence of pests had no significant effect on Aflatoxin level (P-value=0.64).

Discussion

Food contamination with AF is a serious health problem in the community.^[15] Nuts are subjects of this contamination among the food crops because of their composition and storage conditions.^[16]

In the present study, as shown in table 1, total AF level was detected in 11.76% of raw pistachios and in 2.94% of roasted pistachios which were lower than MTL of Iran (15 ppb). reported that the total AF level was detected in 28.3% of pistachios with mean of 7.3 \pm 53.2 µg/kg which was lower than MTL of Iran (15 ppb).

In peanuts, the mean of total AF levels of roasted peanut samples was lower than MTL of Iran (15 ppb).

A study in Zanjan (Iran) indicated that 60% of salted peanuts and 93.7% of pure samples were contaminated with AF, $^{[17]}$ while findings of the present studies showed that 25% of the roasted samples and 23.33% of raw samples were contaminated with AF. Higher levels of AF in the roasted samples might be due to the prolonged storage, ^[18] moisture, ^{[19], [20]} and suitable temperature for growth of mycotoxigenic molds.^[19]

In addition, during the sample roasting procedure, fungi are probably destroyed, but their toxins (AFs) are stable to dry heat, sometimes even up to 250°C.^[21] in Thailand stated that 3.6% of raw groundnuts and 50% of roasted groundnuts had AF contaminations of more than 20 ppb (MTL of AF levels set by the Thailand ministry of public health). According to the findings of various studies, in Iran, long term storage of roasted nuts in bad conditions can cause AF contaminations in market sand stores.

In the Qatar, walnut samples were free from AF^[22] that their findings were closer to our study, while Juan in morocco reported that incidence of AF in walnuts was 30%^[4] and According to the findings of researchers in Pakistan, ^[23] shell-less walnuts had the maximum contamination level among all nut samples.

Giorni *et al.* $(2006)^{[24]}$ reported that moisture content was necessary for growth of mycotoxigenic molds.

High levels of AF in the nuts are probably due to the traditional methods of manufacturing, storage conditions (high humidity and suitable temperature for growth of mycotoxigenic molds), transition and marketing that accelerate fungi growth which resulted in increment of AFs in nuts. It seems that long-term consumption of the AF-contaminated nuts has carcinogenic and toxigenic effects on the human health. Hence necessary steps should be taken by the health organizations and other related agencies to minimize the AF contamination and also train the general population about the threats of AF in nuts. It is recommended that proper storage methods be implemented to control and reduce the contamination by establishment of a systematic food control systems. (i.e. GAP and HACCP) Educating farmers, manufacturers, distributors, retailers and warehouse owners is also essential.

Conclusion

According to the high consumption of nuts and harmful effects of aflatoxin, contamination of nuts with it should be considered as a health hazard, and proper interventions be taken into account.

References

 Stroka J, Anklam E. New strategies for the screening and determination of aflatoxins and the detection of aflatoxin-producing moulds in food and feed. TrAC Trends Anal Chem [Internet]. 2002 Feb [cited 2014 May 27];21(2):90–5. Available from: <u>http://linkinghub.elsevier.com/retrieve/pii/S0165</u> 993601001339.

- 2. David L. Eaton JDG. The Toxicology of aflatoxins:Human health, veterinary and agricultural significance. Academic Press; 1994.
- Dini A, Khazaeli P, Roohbakhsh A, Madadlou A, Pourenamdari M, Setoodeh L, et al. Aflatoxin contamination level in Iran's pistachio nut during years 2009–2011. Food Control [Internet]. 2013 Apr [cited 2014 May 27];30(2):540–4. Available from: http://linkinghub.elsevier.com/retrieve/pii/S0956

<u>http://linkinghub.elsevier.com/retrieve/pil/S0956</u> <u>713512004604</u>.

- Juan C, Zinedine A, Moltó JC, Idrissi L, Mañes J. Aflatoxins levels in dried fruits and nuts from Rabat-Salé area, Morocco. Food Control [Internet]. 2008 Sep [cited 2014 May 27];19(9):849–53. Available from: http://linkinghub.elsevier.com/retrieve/pii/S0956 713507001715.
- 5. Speijers GJA, Speijers MHM. Combined toxic effects of mycotoxins. Toxicol Lett. 2004 Oct 10;153(1):91–8.
- Kujawa 6. М. Some Naturally Occurring Substances: Food Items and Constituents, Heterocyclic Aromatic Amines and Mycotoxins. IARC Monographs on the Evaluation of Carcinogenic Risks to Humans, Vol. 56. Herausgegeben von der International Agency for Research on Cancer. W. Food / Nahrung [Internet]. 1994 [cited 2014 May 27];38(3):351-351. Available from: http://doi.wiley.com/10.1002/food.19940380335
- Moss MO. Risk assessment for aflatoxins in foodstuffs. Int Biodeterior Biodegradation [Internet]. 2002 Oct [cited 2014 May 27];50(3-4):137–42. Available from: http://linkinghub.elsevier.com/retrieve/pii/S0964 830502000781.
- T H E European Commission. COMMISSION REGULATION (EU) No 165/2010 of 26 February 2010 amending Regulation (EC) No 1881/2006 setting maximum levels for certain contaminants in foodstuffs as regards aflatoxins. Official Journal of the European Union; 2010.
- 9. ISIRI (2010). ISIRI. Institute of Standards and Industrial Research of Iran; 2010.
- 10. PITTET A. Natural occurrence of mycotoxins in fonds and feeds an updated review. Rev Med Vet. 1998;6:479–92.
- 11. Evaluation of certain food additives and contaminants. Forty-ninth report of JECFA.. Geneva: World Health Organization; 1998.
- Kashaninejad M, Mortazavi A, Safekordi A, Tabil LG. Some physical properties of Pistachio (Pistacia vera L.) nut and its kernel. J Food Eng [Internet]. 2006 Jan [cited 2014 May

27];72(1):30–8. Available from: http://linkinghub.elsevier.com/retrieve/pii/S0260 877404005898.

- Yazdanpanah H, Mohammadi T, Abouhossain G, Cheraghali AM. Effect of roasting on degradation of Aflatoxins in contaminated pistachio nuts. Food Chem Toxicol [Internet]. 2005 Jul [cited 2014 May 27];43(7):1135–9. Available from: http://www.ncbi.nlm.nih.gov/pubmed/15833389
- Bhat RV and SV. Food Safety in Food Security and Food Trade: Mycotoxin Food Safety Risk in Developing Countries. 2003. International Food Policy Research Institute (IFPRI); 2003.
- 15. A Kazemi1, J Mohtadi Nia2, R Mahdavi2, A Ostad Rahimi2, SJ Ghaem Maghami2, M Vahed Jabbari2, E Ghayour2, M Mohammad Zade3. A Jafari4 A Kazemi1, J Mohtadi Nia2, R Mahdavi2, A Ostad Rahimi2, SJ Ghaem Maghami2, M Vahed Jabbari2, E Ghayour2 MMZAJ. Survey of Food Stuff to Carcinogenic Mycotoxins. The 3rd Congress of MedicalPlants. Tehran; 2007. p. 386.
- Sabaté J, Ang Y. Nuts and health outcomes: new epidemiologic evidence. Am J Clin Nutr [Internet]. 2009 May [cited 2014 May 28];89(5):1643S–1648S. Available from: <u>http://www.ncbi.nlm.nih.gov/pubmed/19321572</u>
- 17. Rostami R, Naddafi K, Aghamohamadi A, Saleh HN DM. Survey of peanut fungal contamination and its relationship with ambient conditions in the Bazar of Zanjan. Iran J Env Heal SciEng. 2009;6(4):295–300.
- Iqbal A, Khalil IA, Shah H. Aflatoxin contents of stored and artificially inoculated cereals and nuts. Food Chem [Internet]. 2006 Jan [cited 2014 May 27];98(4):699–703. Available from: <u>http://linkinghub.elsevier.com/retrieve/pii/S0308</u> <u>814605005716</u>.

- Shakerardekani A, Karim R. Effect of different types of plastic packaging films on the moisture and aflatoxin contents of pistachio nuts during storage. J Food Sci Technol [Internet]. 2013 Apr [cited 2014 May 27];50(2):409–11. Available from: <u>http://www.pubmedcentral.nih.gov/articlerender.</u> <u>fcgi?artid=3550915&tool=pmcentrez&rendertyp</u> e=abstract.
- 20. Youssef MS, El-Maghraby OMO IY. Mycobiota and Mycotoxins of Egyptian Peanut (Arachishypogeae L.) Seeds.. Int J Bot. 2008;4(4):349–360.
- 21. Kladpan S, Mahakachanakul W, Yongmanitchai V, Boonyaratanakornkit M CA . Situation of aflatoxin contamination in groundnut and groundnut products in Thailand in 2004. Proceedings of 43rd Kasetsart University Annual Conference, Thailand, 1-4 February, 2005 Subject: Animals. Thailand; 2005. p. 557–564..
- 22. Abdulkadar AH., Al-Ali A, Al-Jedah J. Aflatoxin contamination in edible nuts imported in Qatar. Food Control [Internet]. 2000 Apr [cited 2014 May 27];11(2):157–60. Available from:

http://linkinghub.elsevier.com/retrieve/pii/S0956 713599000882.

- Luttfullah G, Hussain A. Studies on contamination level of aflatoxins in some dried fruits and nuts of Pakistan. Food Control [Internet]. 2011 Mar [cited 2014 May 27];22(3-4):426–9. Available from: <u>http://linkinghub.elsevier.com/retrieve/pii/S0956</u> <u>713510002926</u>.
- 24. Giorni P, Magan N, Pietri A, Bertuzzi T, Battilani P. Studies on Aspergillus section Flavi isolated from maize in northern Italy. Int J Food Microbiol [Internet]. 2007 Feb 15 [cited 2014 May 27];113(3):330–8. Available from: http://www.ncbi.nlm.nih.gov/pubmed/17084935.

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