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Qualitative and Quantitative Analysis of Aflatoxins in Dry Fruits and Nuts from Central India

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ABSTRACT

In central part of India, dry fruits and nuts are mostly sold in loose conditions and hence may get fungal infections, and may have aflatoxins to the levels beyond the safe limits. The present study is an attempt to showcase the present scenario of aflatoxins in dry fruits and nuts from Central India, both qualitatively and quantatively. Out of 84 samples, 15 were found to have the presence of at least one aflatoxin. HPLC detection showed that AFB_1 is the major aflatoxin. The total aflatoxins were found in the range of 25.4 - 393.51 µg kg^{-1}, which is beyond the safe limit set by regulatory authorities. Further, dry figs were the most contaminated among tested dry fruits, and 5 out 12 samples (41.6%) were contaminated, followed by cashew nuts (33.3%). Samples sold in loose packing were found more contaminated with aflatoxins. The study advocates that selling of such dry fruits in loose form should be banned by the regulatory authorities.

Keywords: Fungi; Dry fruits; Mycotoxin; Aflatoxin; HPLC

1. INTRODUCTION

Mycotoxins are toxic compounds produced by fungal growth in food substances. Although not all fungi produce toxins, mycotoxins have a great economic impact on food production, because of the damage of feedstuffs and impairment of food quality. The most significant mycotoxins affecting food are aflatoxin, vomitoxin, zearalenone, fumonisin, and ochratoxin. These mycotoxins are produced primarily by fungi belonging to the genera *Aspergillus*, *Fusarium*, and *Penicillium*[1,2].

Mycotoxins are serious health concerns to the humans, as they can cause acute or chronic ill effects. The mycotoxins can be present in various food commodities and are defined as a major food safety concern all over the world[3]. Since, there are geographic and climatic differences in the production and occurrence of mycotoxins, monitoring for the presence of mycotoxins at local levels is therefore always needed[4]. Researchers have found it impossible to completely remove the mycotoxins from foodstuffs; hence guideline for the allowed maximum level has been adopted. These maximum residue levels are different for different categories of food, and include dry fruits and nuts too. This allowable limit is based on the incidences of occurrence of aflatoxins in different foods, as well as on the present perception of safety guidelines pertaining to the exposure to these aflatoxins[5].

Among mycotoxins, aflatoxins are more abundant toxins and carcinogens, contaminating oilseeds, spices, dry fruits and tree nuts, cereals and grains[6]. Exposure to aflatoxin may cause acute hepatic necrosis, followed by cirrhosis of the liver. There is no specific antidote for aflatoxicosis[7]. The *Aspergillus*, especially the members of section *Flavi*, have been shown to produce aflatoxins, and the food commodity can be contaminated at almost every stage of the food chain, pre-harvest, post harvest, processing, transportation and storage[1].

Dry fruits and nuts are costly food stuffs known not only for their taste and flavor but for instant sources of energy, as well as antioxidants. Since, dry fruits and nuts are consumed directly most of time, there is a higher chance of exposure to the aflatoxins. This makes sense of analyzing the aflatoxin quantities during each step of storage, handling, drying and packaging processes of such priced commodities[8]. Therefore, realizing the potential hazards and economic values of dry fruits, the present study is proposed to study the occurrence and hazard analysis study on aflatoxins in commercial grades dry fruits and nuts being sold in Jabalpur, Madhya Pradesh India.

2. MATERIALS AND METHODS

2.1 Sample Collection

To study the status of fungal contaminations in dry fruit and nut products, the samples chosen were the major dry fruits being consumed in Jabalpur. These included; cashew nuts, raisins, almonds, dried figs, walnuts, dry dates, and dried apricots. The samples were collected or purchased randomly from the different local grocery stores of Jabalpur. Both loose and packed dried fruits were collected for the study. Twelve samples (one sample per month) each of seven most used dry fruits and nuts were collected during one year

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time. Peanuts and popped lotus seeds are not included in the study as they belong to different food category by FSSAI, Govt. of India.

2.2 Extraction of Aflatoxins

All the samples were dried completely before extraction. Fifty gram portion of a ground sample was mixed with 25 g Celite and 25 ml distilled water. The aflatoxin from the slurry thus prepared was extracted with 250 mL chloroform, using wrist action shaking for 30 min. The suspension was allowed to settle for a while and filtered through a fluted filter paper (Whatman No. 1). First 50 mL of filtrate was collected.

For further sample cleanup, the extract was loaded onto a silica gel column, prepared in chloroform, via an anhydrous sodium sulphate bed. The column was washed with 150 mL *n*-hexane, and then with 150 mL petroleum ether. Aflatoxins were eluted with 150 mL methanol–chloroform (3:97 v/v). The eluate was concentrated on a water bath at 40°C, and the residue was made up to 1.0 mL with chloroform[9].

2.3 Qualitative Estimation of Mycotoxins by Thin Layer Chromatography (TLC)

For qualitative estimations of aflatoxins, known amount of sample extracts (10µl) were spotted on a line 2 cm above the bottom of activated Silica gel G TLC plates and developed with a solvent system consisting of acetone: chloroform (1:9 v/v). Developed TLC plates were examined under long wave UV light (365 nm) and the pattern of the four fluorescent spots, corresponding to four aflatoxins was observed[10]. A standard aflatoxin mix (Sigma Aldrich, USA) is also run side by-side for comparison purposes.

2.4 Quantitative Estimation of Aflatoxins by HPLC

All reagents for the mobile phase used were HPLC grade from Merck-Millipore Ltd. (Germany). The samples showing at least one fluorescent spot upon thin layer chromatography were further resolved and quantified using the High Performance Liquid Chromatography (HPLC) with ultraviolet detection at 365 nm[11]. The HPLC (Shimadzu, Japan) was set at 365 nm with a reverse-phase ODS C18 column (Shim-pack MAqC-ODS; 4.6 x 250 mm, 5 µm) under a 20 °C controlled column chamber. The samples were injected using an automated sample injector kept at 4°C.

For quantification, a standard calibration curve was prepared using certified reference material for aflatoxins, containing AFB1, AFB2, AFG1 and AFG2 (Sigma Aldrich, USA). Aflatoxin certified reference materials were diluted to get 1 ppm working solutions. The run conditions were: sample injection volume 10 μl; mobile phase water: methanol: acetonitrile (63:26:11) pH 6.8; flow rate 1.0 mL min^{-1}; pressure 400 bar; detector 365 nm, detector flow cell temperature 20 °C; and run time 35 min. Aflatoxins were detected and quantified using the retention time as marker and compared with that of the standard aflatoxin mixture.

Calculations:

Individual aflatoxin concentration was calculated as follows:

Aflatoxin (µg kg^{-1}) = (A/ A') x C x (2 /10) x 1000 x D

where A and A' = peak areas or heights for test solution and standard, C = concentration of individual aflatoxin in standard solution, and D = dilution factor.

3. RESULTS

In this study, 84 samples, belonging to 7 types of dried fruits and nuts sample were screened for the presence of aflatoxins. Statistical analysis shows that there is a significant difference in fungal contamination in loose conditions as compared to packed ones (Student's *t* test, t=12.48, df=6, $p<0.001$).

For the preliminary idea about the possibility of aflatoxin presence, the samples were chromatographed via thin layer chromatography. The sample bands were compared with those of the standard aflatoxin mix. In aflatoxin standard mixture, AFG_1 and AFG_2 appeared as greenish fluorescent bands with R_f values of 0.72 and 0.64 respectively, while AFB_1 and AFB_2 appeared as blue fluorescent bands with R_f values of 0.91 and 0.84 respectively. Out of 84 samples, 15 samples were positive showing the presence of at least one aflatoxin (Table 1). AFB_1 was the most abundant aflatoxin detected and was present in 11 out of 15 samples (73.3%), followed by AFB_2 (26.6%).

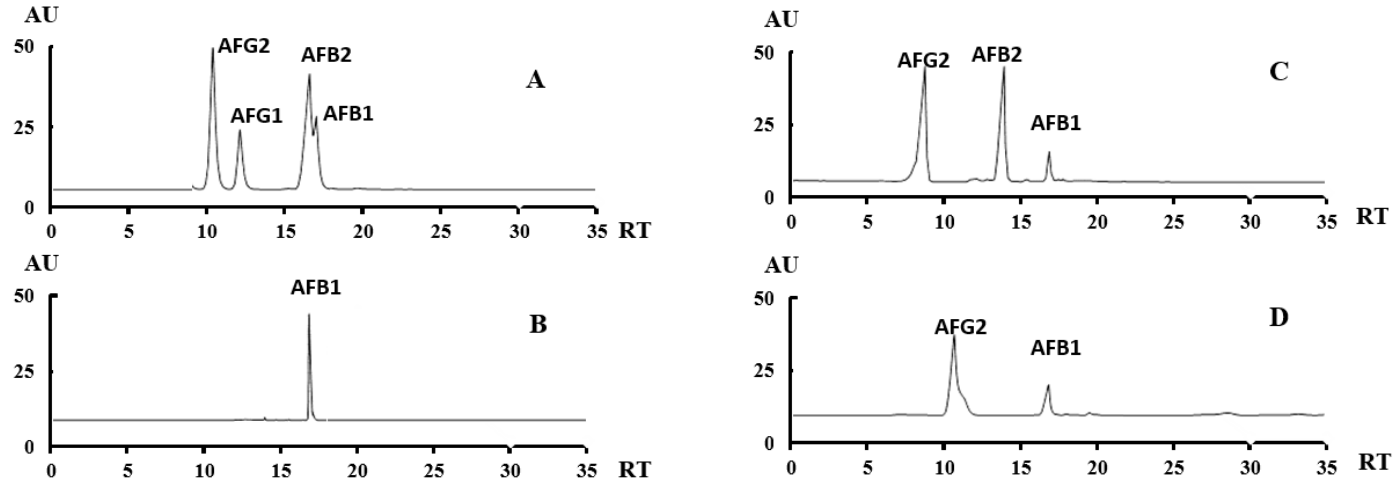
Table 1. Qualitative tests for the presence of aflatoxins by thin layer chromatography in various dry fruit samples collected during 2017-18 from Jabalpur, India.

Sample name	Date of sample collection	Aflatoxins			
		AFG_2	AFG_1	AFB_2	AFB_1
Walnut	07.06.17	-	+	+	-
Dried figs	25.06.17	-	-	-	+
Dried Figs	02.07.17	-	-	-	+
Cashew nuts	04.07.17	-	-	-	+
Cashew nuts	14.11.17	-	-	-	+
Dried figs	20.07.17	-	-	+	+
Almonds	25.07.17	-	-	-	+
Cashew nut	01.08.17	-	-	-	+
Raisin	01.08.17	-	-	-	+
Dried fig	18.09.17	-	-	+	-
Raisin	27.09.17	-	-	-	-
Raisin	23.01.18	-	-	-	+
Cashew nuts	14.04.18	-	-	+	+
Dried figs	07.02.18	-	+	-	-
Apricot	21.05.18	-	-	-	+

The samples found positive for the presence of aflatoxins in TLC, were further purified and chromatographed on High Performance Liquid Chromatography. The aflatoxins were identified and quantified by comparing the relative peak area achieved by the standard aflatoxin mixture.

Figure 1 shows the chromatogram of four aflatoxins in the aflatoxin standard mixture. AFG2 resolved at the retention time of 10.02 min, AFG1 at 11.23 min, AFB2 at 15.87 min and AFB1 at 16.21 min. The standard aflatoxin mixture contained 10 µg each of AFG2 and AFB2 while AFG1 and AFB1 were in the concentration of 25 µg each. It must be noted that the chromatographic profile shows that not all the aflatoxins are present all the time. A higher diversity on aflatoxins was observed.

Table 2 shows that quantification of individual, as well as total aflatoxins in the samples of dry fruits and nuts collected from Jabalpur, India. The results show that AFB1 carries the



Where, RT = Retention Time, AU= Absorbance Unit, AF= Aflatoxin, AFB1= Aflatoxin B1, AFB2 = Aflatoxin B2, AFG1 = Aflatoxin G1, AFG2 = Aflatoxin G2

Figure 1. HPLC chromatogram showing the presence of various aflatoxins from A: Certified aflatoxin mixture, B: Dry fig 2.7.17 C: Dry fig 20.7.2017 and D: Raisin 21.1.2018 collected from Jabalpur, India.

Table 2. Quantification of aflatoxins in various dry fruit samples collected during 2017-18 from Jabalpur

Sample name	Date of sample collection	aflatoxins (µg kg^{-1})				Total aflatoxin (µg kg^{-1})
		AFG_2	AFG_1	AFB_2	AFB_1	
Walnut	07.06.17	-	142.86	43.76	-	186.62
Dried figs	25.06.17	-	-	-	33.20	33.20
Dried figs	02.07.17	-	-	-	109.24	109.24
Cashew nuts	04.07.17	-	-	-	113.45	113.45
Cashew nuts	14.11.17	-	-	-	393.51	393.51
Dried figs	20.07.17	105.51	-	140.03	53.20	298.74
Almonds	25.07.17	-	3.76	-	21.64	25.40
Cashew nut	01.08.17	-	-	-	132.20	132.20
Raisin	01.08.17	78.09	26.82	-	-	104.91
Dried fig	18.09.17	-	-	42.87	-	42.87
Raisin	27.09.17	-	137.60	-	-	137.60
Raisin	23.01.18	21.63	-	-	40.47	62.10
Cashew nuts	14.04.18	-	-	94.12	31.08	125.20
Dried figs	07.02.18	-	21.58	-	-	21.58
Apricot	21.05.18	-	-	-	35.01	35.01

major portion of total aflatoxins. The total aflatoxins were found to be in the range of 25.4 - 393.51 µg kg^{-1}. Further, dry figs were the most contaminated among tested dry fruits, and 5 out 12 samples (41.6%) were contaminated, followed by cashew nuts (33.3%). Samples sold in loose packing were found more contaminated with aflatoxins.

4. DISCUSSION

Due to the common practice to sell dry fruits in loose conditions, these food stuffs are highly vulnerable for fungal attack including the toxigenic fungi. Further, the Food Safety and Standards Authority of India (FSSAI) has food safety norms only for nuts and dry figs among the dry fruits, and that too only, in the presence of total aflatoxins. The present study is oriented towards identifying the diversity of aflatoxins in various dry fruits that were collected from Jabalpur, Central India; both in loose and packed conditions. The present study was aimed to identifying the diversity of aflatoxins in various dry fruits that were collected from Jabalpur, Central India; both in loose and packed conditions. Significant variations in types and amount of aflatoxins were recorded in various samples. However, except 15 samples, the aflatoxins were below the detectable limits. All the four major types of aflatoxins were present. Earlier, aflatoxins have been reported in various food commodities in India[12]. Aflatoxins B_1, B_2, G_1, G_2 are the most toxic and carcinogenic naturally occurring mycotoxins. Due to their extreme hepatocarcinogenicity, extensive research has been carried out on the natural occurrence, identification, characterisation, biosynthesis, and genetic regulation of aflatoxins[13,14].

Study shows that most of the samples had higher aflatoxin concentrations, beyond the safe limits specified by FSSAI in India. Dry figs were especially contaminated by the aflatoxins. Variations in sugar and moisture content may be the major factors for difference in the occurrence of aflatoxins in the samples. Contamination of figs with aflatoxins begins during sun drying on the tree and enhanced by the unhygienic environment during handling[15]. Levels can be very high: up to 76000 µg kg^{-1} AB_1 (measured by TLC)[16], and up to 72 µg kg^{-1} aflatoxin B_2 (AB_2)[17]. Recently, it was noted that dry

figs, claimed to be safe from aflatoxins, had presence of other mycotoxins, i.e. kojic acid[18]. AFB_1 has also been found in dried apricots[19], pistachio, walnut and almonds[20], and dates[21].

In India, FSSAI has set the maximum level of total aflatoxins in nuts as 15 µg kg^{-1} and 10 µg kg^{-1} in figs. The regulations in India have not prescribed the limit for individual aflatoxins, as well as other mycotoxins, in other dry fruits such as raisins and dates. The present study shows that raisins and other dry fruit may also contain aflatoxin levels well beyond the safe limit. Further, in India, most of the dry fruits are sold in loose conditions, where unhygienic storage and hand handling may promote fungal growth. Our study also supports this notion, as loose samples were more contaminated with aflatoxins in comparison to the packed samples. Hence, based on our study, it can be advised that more stringent rules need to be applied to dry fruit producers, handlers, and retailers for the food safety purposes.

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REFERENCES

1. Frisvad, J.C.; Hubka, V.; Ezekiel, C.N.; Hong, S.B.; Nováková, A.; Chen, A.J.; Arzanlou, M.; Larsen, T.O.; Sklenář, F.; Mahakarnchanakul, W. & Samson, R.A. Taxonomy of Aspergillus section Flavi and their production of aflatoxins, ochratoxins and other mycotoxins. *Studies Mycology*, 2019, **93,** 1-63. doi: 10.1016/j.simyco.2018.06.001
2. Ismail, A.; Riaz, M.; Gong, Y.Y.; Akhtar, S. & Sun, J. Aflatoxins in plant-based foods. *Plant Human Health,* 2019, **2,** 313-325. doi: 10.1007/978-3030-03344-6_13
3. Kuiper-Goodman, T. Risk assessment & risk management of mycotoxins in food. In *Mycotoxins in food*. 2004, 3-31. Woodhead Publishing. doi: 10.1533/9781855739086.1.3
4. Kos, J.; Hajnal, E.J.; Šarić, B.; Jovanov, P.; Nedeljković, N.; Milovanović, I. &Krulj, J. The influence of climate conditions on the occurrence of deoxynivalenol in maize harvested in Serbia during 2013–2015. *Food Control*, 2017, **73,** 734-740. doi: 10.1016/j.foodcont.2016.09.022
5. Tulayakul, P. & Sugita-Konishi, Y. Mycotoxin contamination in foodstuffs & feeds-health concerns in Thailand. *Japanese J. Veterinary Res.*, 2017, **65**(3), 173-183. doi: 10.14943/jjvr.65.3.173
6. Smith, M.C.; Madec, S.; Coton, E. & Hymery, N. Natural co-occurrence of mycotoxins in foods and feeds and their in vitro combined toxicological effects. *Toxins*, 2016, **8**(4), 94. doi: 10.3390/toxins8040094
7. Brown, L.R. Aflatoxins in food & feed: Impacts risks, and management strategies. *Int. Food Policy Res. Inst.* 2018, **9.** doi: 10.2499/1046080706
8. Medina, A.; Gilbert, M.K.; Mack, B.M.; OBrian, G.R.; Rodriguez, A.; Bhatnagar, D.; Payne, G. & Magan, N. Interactions between water activity and temperature on the Aspergillus flavus transcriptome and aflatoxin B1 production. *Int. J. food Microbiol.*, 2017, **256,** 36-44. doi: 10.1016/j.ijfoodmicro.2017.05.020
9. FSSAI Manual of Methods for Mycotoxins. Food Safety &Stanards Authority of India (FSSAI), 2016, Government of India.
10. Zahra, N.; Khan, M.; Mehmood, Z.; Saeed, M.K.; Kalim, I.; Ahmad, I. & Malik, K.A. Determination of aflatoxins in spices and dried fruits. *J. Sci. Res.*, 2018, **10**(3), 315-321. doi: 10.3329/jsr.v10i3.37075
11. Fu, Z.; Huang, X. & Min, S. Rapid determination of aflatoxins in corn and peanuts. *J. Chromatography A*, 2008, **1209**(1-2), 271-274. doi: 10.1016/j.chroma.2008.09.054
12. Aiko, V. & Mehta, A. Prevalence of toxigenic fungi in common medicinal herbs and spices in India. *3 Biotech*, 2016, **6** (2), 159. doi: 10.1007/s13205-016-0476-9
13. Heshmati, A.; Zohrev, 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. doi: 10.1016/j.fct.2017.05.046
14. Škrbić, B.; Antić, I. & Cvejanov, J. Determination of mycotoxins in biscuits, dried fruits and fruit jams: an assessment of human exposure. *Food Additives Contaminants: Part A*, 2017, **34** (6), 1012-1025. doi: 10.1080/19440049.2017.1303195
15. Ait Mimoune, N.; Arroyo-Manzanares, N.; Gámiz-Gracia, L.; García-Campaña, A.M.; Bouti, K.; Sabaou, N. & Riba, A. *Aspergillus* section *Flavi* & aflatoxins in dried figs and nuts in Algeria. *Food Additives Contaminants: Part B*, 2018, **11** (2), 119-125. doi: 10.1080/19393210.2018.1438524
16. Bakirci, G.T. Investigation of aflatoxins levels in commercial dried figs from western Turkey. *Int. Food Res. J.*, 2020, **27**(2), 245-251.
17. Sulyok, M.; Krska, R. & Senyuva, H. Profiles of fungal metabolites including regulated mycotoxins in individual dried Turkish figs by LC-MS/MS. *Mycotoxin Research*, 2020, 1-7. doi: 10.1007/s12550-020-00398-5
18. Basegmez, H.I.O. Dietary Exposure Assessment of Aflatoxin From Dried Figs in Turkey. *Hittite J. Sci. Eng.*, 2019 **6**(3), 173-177. doi: 10.17350/HJSE19030000144
19. Gupta, D.; Bala, P. & Sharma, Y.P. Evaluation of fungal flora & mycotoxin contamination in whole dried Apricots (Prunus armeniaca L.) from J&K, India. *In* Proceedings of the National Academy of Sciences, India Section B:

Biological Sciences. 2015, **87** (1), 81-87.
doi: 10.1007/s40011-015-0589-7

20. Shakeri, Z.; Rahimi, E. & Shakerian, A. Evaluation of aflatoxin content in pistachio, almond, hazelnut and walnut in Isfahan. *J. Food Hygiene* 2019, **9, 2** (34), 61-69.
21. Nasser, L.A. Fungal contamination and invertase activity in dates and date products in Saudi Arabia. *American J. Food Technol.*, 2017, **12**, 295-300.
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