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AFLATOXIN CONTAMINATION IN CHILGOZA PINE NUTS (*Pinus gerardiana* Wall.) COMMERCIALLY AVAILABLE IN RETAIL MARKETS OF JAMMU, INDIA

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ABSTRACT

The aim of the present investigation was to check natural aflatoxin contamination and aflatoxigenic potential of *Aspergillus flavus* strains isolated from chilgoza pine nuts. Fifty eight samples of chilgoza pine nuts, randomly purchased from retail shops and local markets of different locations of Jammu, were subjected to mycological analysis for isolation of *Aspergillus flavus* isolates using standard blotter method. A total of 35 isolates were recovered during mycological analysis of these nuts. Of these 25.71% isolates were aflatoxigenic, producing aflatoxin B_1 and B_2 in varying amount. Natural contamination of aflatoxins was detected in 46.55% of investigated samples (58) with a mean range of 0.737±0.023-1.675±0.486µg/g, which is quite high in comparison to maximum tolerable limits of 4µg/kg set forth by the European Union Commission for ready to eat nuts. The present study is the first report on the incidence of aflatoxin contamination in chilgoza from the markets of Jammu, India.

KEYWORDS: **Aflatoxin, *Aspergillus flavus*, Chilgoza, HPLC, TLC**

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INTRODUCTION

Aflatoxins (AFs) are a group of naturally occurring and the most toxic class of mycotoxins. Chemically, they are difuranocoumarin derivatives produced by a polyketide pathway by many strains of *Aspergillus flavus*, *Aspergillus parasiticus* and a number of other species. Currently, twenty species of *Aspergillus* have been known to produce aflatoxins. Out of these, 15 species belong to section *Flavi* (*Aspergillus flavus*, *A. parasiticus*, *A. arachidicola, A. novoparasiticus*, *A. sergii*, *A. transmontanensis, A. nomius, A. parvisclerotigenus, A. minisclerotigenes, A. pseudocaelatus*, *A. tamari*, *A. bombycis*, *A. pseudonomius*, *A. mottae* and *A. togoensis*). Two species belong to section *Ochraceorosei* (*A. ochraceoroseus* and *A. rambellii*) while the remaining three are related to section *Nidulantes* (*Emericella astellata*, *E. venezuelensis* and *E. olivicola*) [1-13]. There are about 20 known structurally related aflatoxins out of which four i.e. aflatoxin B_1 (AFB_1), B_2 (AFB_2), G_1 (AFG_1) and G_2 (AFG_2) are the major naturally produced aflatoxins and their order of toxicity is $AFB_1 > AFG_1 > AFB_2 > AFG_2$ [14]. They are highly thermostable and survive food processing and, therefore, can enter food chain. Ingestion of aflatoxin B_1 and B_2 contaminated food by lactating humans and animals lead to their biotransformation into hydroxylated forms M_1 and M_2 which are excreted in their milk. Contamination by aflatoxins (AF) is a major problem for tree nuts, as well as for other stored grains, milk and dry fruits, especially because the causal fungi, *Aspergillus flavus* group, occur as a natural contamination [15,16] . Chilgoza pine nuts, commonly referred to as chilgoza, are edible seeds of chilgoza pine (*Pinus gerardiana* Wall.) belonging to family Pinaceae. This species is a native of North-West Himalayas and is sparsely distributed in dry rocky slopes extending from north-east Afghanistan, Tibet, Pakistan (Baluchistan), China and north India [17-18]. Chilgoza pine nuts form an important international trade commodity consumed worldwide as raw, roasted or as an ingredient in desserts. Many value added products such as nut butter, nut powder, nut toffee, brittle caramel and other sweet candies are manufactured with chilgoza kernels [19]. In addition, the seeds possess vital medicinal attributes being carminative, stimulant, expectorant and aphrodisiac [20,21]. In India, surveys on the mycotoxin contamination particularly aflatoxins have been taken on various dry fruits and dried fruits but a little attention has been paid towards chilgoza pine nuts [16,22,23,24]. In view of this, an investigation was undertaken (a) to investigate the incidence of *Aspergillus flavus* in chilgoza pine nuts; (b) evaluating the ability of *Aspergillus flavus* to produce aflatoxins; and (c) determination of natural occurrence of aflatoxins in market samples of chilgoza pine nuts

MATERIALS AND METHODS

Chilgoza samples were randomly obtained from different retailers of dry fruit markets of five districts (Jammu, Kathua, Kishtwar, Samba and Udhampur) of Jammu province (J&K) during March-December, 2011. The samples were collected in sterilized polyethylene bags to avoid further contamination and stored in refrigerator at 5°C till further studies.

(i) Chemicals and reagents

Aflatoxin B_1 and B_2 standards were purchased from Hi-Media, Mumbai. Stock solutions of aflatoxins were prepared in HPLC grade methanol and kept in darkness at -20°C. Working solutions were prepared immediately before use by diluting stock solution with HPLC grade methanol. Solvents used for High performance liquid chromatography were of HPLC grade while all other reagents used were of the highest analytical grade available. Five point calibration curves, with triplicate injection, at 5, 25, 50, 75, 100ng/ml for both aflatoxin B_1 and B_2 were constructed to calibrate High

Performance Liquid Chromatography (HPLC) instrument.

(ii) Mycological analysis for isolation of Aspergillus flavus isolates

Aspergillus flavus isolates were obtained by standard blotter method recommended by International Seed Testing Association[25]. Colonies were individually sub-cultured on Czapek-Dox agar (CDA) and Malt extract agar (MEA) at 28±2°C. Identification was done by studying cultural and micro-morphological characters following Raper and Fennell [26] and Pitt and Hocking [27].

(iii) Screening of toxigenic strains of Aspergillus flavus recovered from market samples of chilgoza pine nuts

Aflatoxin producing potential of *Aspergillus flavus* isolates obtained from market samples of chilgoza were tested in rice flour liquid medium (rice flour, 40.0g; sucrose, 30.0g; yeast extract, 2.0g, streptomycin sulphate, 0.06g; distilled water, 1000ml) following Misra and Sinha [28]. Erlenmeyer flasks (250ml) containing 100ml of autoclaved rice flour medium were inoculated with 1ml of spore suspension (~10^6 spores per ml) from 7 day old cultures of *A. flavus*. These flasks were incubated at 28±2°C for 10 days and manually shaken twice a day. After incubation, the content of the flask were filtered through Whatman no. 1 filter paper. The filtrate was extracted thrice using chloroform (total volume 50ml) in a separating funnel. The separated chloroform extract was evaporated to dryness over water bath. The residue left after evaporation was dissolved in 2ml chloroform and stored in vials at -20°C in the darkness for qualitative and quantitative analysis.

(iv) Extraction of aflatoxins from naturally contaminated chilgoza pine nuts

For detection and estimation of aflatoxins in naturally contaminated market samples of chilgoza, method of Thomas *et al* [29] was employed. 50g each of dried finely crushed sample was taken in 500ml Erlenmeyer flask containing 250ml mixture of methanol and water (60:40v/v) and shaken on horizontal rotary shaker for 30 minutes. Thereafter, the mixture was filtered and 125ml filtrate was taken in 250ml separating funnel along with 50ml of n-hexane and shaken vigorously for two minutes. Discarded the upper layer and lower methanol layer was taken in another 250ml separating funnel. To this, added 50ml chloroform and shaken vigorously. Lower chloroform layer was collected in a conical flask containing 5g cupric carbonate, shaken and vacuum filtered through Whatman no. 42 filter paper having a bed of anhydrous sodium sulphate. The extract so obtained was collected in a beaker. Cupric carbonate was again washed through sodium sulphate bed. The two chloroform extracts, thus, obtained were pooled and concentrated to dryness over the water bath. The residue was dissolved in 2ml chloroform and stored in vials at -20°C in the darkness for qualitative and quantitative analysis of aflatoxins. All the procedures were carried out in subdued light since aflatoxins are subjected to light degradation.

(v) Qualitative and quantitative estimation of aflatoxins

Qualitative estimation of aflatoxins was done by thin layer chromatography (TLC). Known aliquots of chloroform extract of samples to be assayed were spotted on a line 2cm above the bottom line of activated TLC plates along with standard solutions, using micropipette. The spotted plate was developed in the solvent system consisting of toluene: iso-amyl alcohol: methanol (90:32:2v/v/v) in a sealed TLC tank. After developing, these plates were air dried and observed under long wave UV light (366nm). The chemical confirmation of aflatoxins was done by spraying 25% H_2SO_4 which changed the blue fluorescent spots to yellow[29]. Aflatoxins were determined visually by comparing Rf values and colour intensities of the samples with the aflatoxin standards under ultraviolet light. Quantitative estimation of aflatoxins was done through High Performance Liquid Chromatography (HPLC) by standardizing a novel method based on the methodology described by Sigma-Aldrich [30].

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The analytical equipment for HPLC (CLASS-LC 10 SHIMADZU) consisted of a liquid chromatographic pump LC-10AT, an auto-injection system SIL-10A with a 50µl sample loop, a variable wavelength absorbance detector SPD-10, reverse phase analytical column C-18 (250 x 4mm) filled with ODS (M), RP-18 material, 5µm particle size (Merck). Each extract was filtered with 0.45µm membrane filter and then transferred into HPLC vials for auto injection. Injection volume of extract solution was 30µl. Isocratic elution was done with water: acetonitrile: methanol (54:34:12v/v/v) at a flow rate of 1.5ml/minute. A variable wavelength UV-VIS detector set at 365nm was used. Quantification was done by comparison of retention time (aflatoxin B_1- 5.26 minutes and B_2- 4.55 minutes) and peak area observed in the aflatoxin standard solution with those observed in the samples (Figure1). Analysis was performed at room temperature and recorded in HP DeskJet 670C.

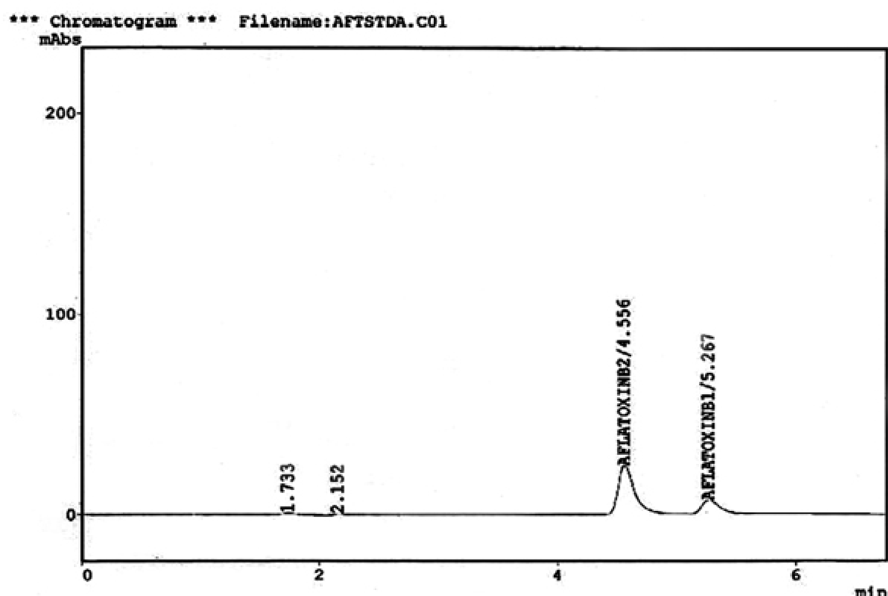

Figure 1
HPLC chromatogram of Aflatoxin standard B_1 and B_2

(vi) Statistical analysis
The statistical significance of the experiments was analyzed by one way analysis of variance (ANOVA) with Tukey's test of significance. Statistical analysis was performed by using IBM SPSS-20.0, software. Differences among sample mean were reported to be significant when $p<0.05$.

RESULTS AND DISCUSSION

(i) Mycological analysis and aflatoxigenic potential of Aspergillus flavus isolates
A total of 35 isolates of *Aspergillus flavus* were recovered from 58 market samples of chilgoza. Of these, nine (25.71%) isolates were found to be toxigenic and produced aflatoxins in the liquid medium. Eight isolates produced both aflatoxin B_1 and B_2 while one isolate elaborated aflatoxin B_1 only. Comparatively, the amount of aflatoxin B_1 produced by these toxigenic isolates was higher than aflatoxin B_2 (Table1). The overall range of aflatoxins was 6.1500±2.7504 to 73.308±28.112µg/ml. Aflatoxin producing potential of all the toxigenic isolates varied significantly at $p< 0.05$.The behaviour of these isolates in producing different levels of aflatoxins might be due to the genetic makeup of the substrate i.e. host, moisture stress and/ or co-invading fungal

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partners as denoted by earlier researchers [32-34].

(ii) Natural aflatoxin contamination of chilgoza pine nuts

The data presented in the table 2 show that out of the fifty eight samples analyzed, twenty seven samples (46.55%) were positive for aflatoxins B_1 and B_2 contamination. Eleven samples collected from various regions contained aflatoxins B_1 (0.734-1.640μg/g) with a mean value of 0.908±0.310μg/g while thirteen samples (22.41%) were contaminated with aflatoxin B_2 and its concentration ranged from 0.702-2.262μg/g with a mean value of 1.01±0.558μg/g. Additionally, three samples (5.17%) also showed the co-occurrence of aflatoxins B_1 and B_2 (Figure 2). Comparative analysis of aflatoxin contamination in samples collected from various districts showed considerable variation. Udhampur samples were highly contaminated followed by samples collected from Kathua, Samba, Jammu and Kishtwar districts. Level of contamination was considerable high in the samples obtained from Udhampur district. However, the incidence of aflatoxin contamination (number of contaminated samples) was least in the samples collected from this district. This variation may be due to difference in environmental conditions and different types of storage practices. Districts with hot and humid conditions (Samba, Kathua and Jammu) accounted for higher percentage incidence of aflatoxin contamination. The high level of aflatoxin contamination can be explained on the ubiquitous prevalence of toxigenic *Aspergillus flavus* as a natural contaminant in the chilgoza samples examined. *Aspergillus flavus*, in spite of being a weak plant pathogen lacking the ability to penetrate the shell of nuts [35], can gain entry through fairly hard testa into the edible kernel on account of mechanical or biological injuries inflicted by abrasions or insects during development, extraction, transportation and storage of seeds. These results are in consonance with previous reports on Brazil nuts and pistachios [36, 37]. It has been established that the conidia of aflatoxigenic *Aspergillus flavus* strains possess concentration of aflatoxins upto 1g/kg[38] and entry of these conidia via these injuries into the seeds containing nutritionally rich (high fat and carbohydrates) kernels provide a good substrate for colonization and elaboration of aflatoxins by *Aspergillus flavus.*

Table 1
Production of aflatoxins by A. flavus isolates isolated from chilgoza pine nuts

S. No.	Name of Isolate	Districts	Total aflatoxin (μg/ml)	Aflatoxin B_1 (μg/ml)	Aflatoxin B_2 (μg/ml)
1	AF 1	Jammu	*73.308 ± 28.11	*136.170 ± 0.193	*10.446 ± 0.063
2	AF 2	Kathua	24.880 ± 7.07	40.700 ± 0.330	9.060 ± 0.030
3	AF 3	Kathua	44.738 ± 15.80	80.073 ± 0.250	9.403 ± 0.106
4	AF 4	Kathua	70.441 ± 27.28	131.460 ± 0.266	9.423 ± 0.014
5	AF 5	Jammu	28.520 ± 8.51	47.566 ± 0.333	9.473 ± 0.136
6	AF 6	Samba	6.150 ± 2.75	12.300 ± 0.051	-
7	AF 7	Kishtwar	26.681 ± 7.87	44.280 ± 0.238	9.083 ± 0.039
8	AF 8	Jammu	33.181 ± 10.74	57.200 ± 0.115	9.163 ± 0.085
9	AF 9	Kishtwar	29.116 ± 8.95	49.130 ± 0.090	9.10 ± 0.60
Overall Range			**6.150 - 73.308**	**12.300 - 136.170**	**9.083 - 10.446**

**** Values expressed as mean ± S.E***

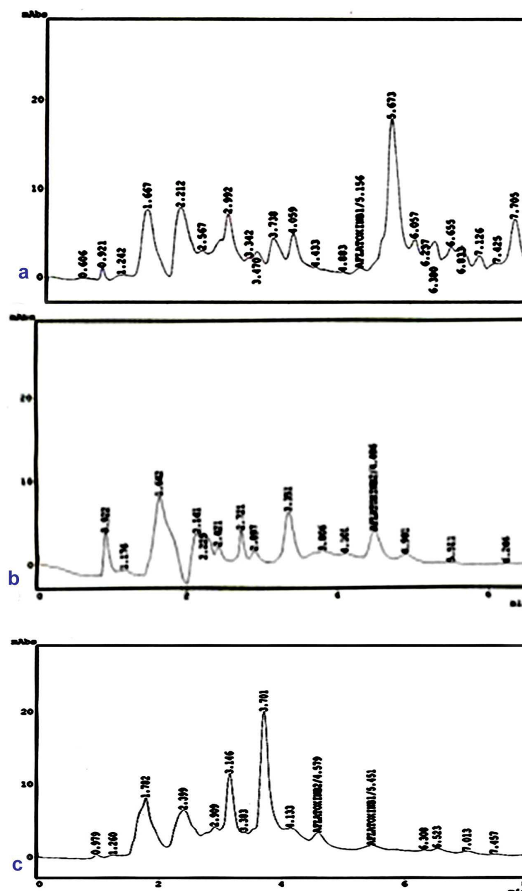

Figure 2
HPLC chromatograms of aflatoxin in market samples of chilgoza pine nuts a.contaminated with aflatoxin B_1 b. contaminated with aflatoxin B_2 c. contaminated with both aflatoxin B_1 and B_2

Aspergillus flavus, moreover, is abundant in tropical environments and has a particular preference for nuts as the substrate[27]. Therefore, aflatoxins are frequently associated with high carbohydrate and high fat food and feed like peanuts and their derived products, almonds, pistachios, Brazil nuts and figs [15, 39-42].

Table 2
Natural aflatoxin contamination in Chilgoza pine nut samples

S. No.	Districts	Number of samples screened	Number of aflatoxin positive samples	Mean aflatoxin contamination (µg/g)	Range of aflatoxin concentration (µg/g)
1	Jammu	19	10 (52.63%)	*0.910 ± 0.158	0.725 - 2.331
2	Kathua	11	6 (54.54%)	1.291 ± 0.335	0.736 - 2.438
3	Kishtwar	12	4 (33.33%)	0.737 ± 0.023	0.702 - 0.806
4	Samba	06	4 (66.66%)	0.979 ± 0.218	0.720 - 1.633
5	Udhampur	10	3 (30.0%)	1.675 ± 0.486	0.702 - 2.17

**** Values expressed as mean ± S.E***

All the aflatoxin contaminated chilgoza samples were found loaded with aflatoxins exceeding the maximum tolerable limit (MTL) of 4µg/kg set by the European Union Commission for ready-to-eat nuts [43]. The levels of contamination also do not comply with the maximum permissible limit of 20ppb aflatoxins set forth by the World Health Organization and less than 30ppb permitted in India [44, 45], thus warranting the attention of health authorities in India, in general, and the state of Jammu and Kashmir, in particular. Survey of literature reveals that the present study constitutes hitherto the first comprehensive report on aflatoxin contamination of chilgoza from the northern region of India excepting for a preliminary report that appeared in two different research journals on aflatoxin contamination in an unspecified number of chilgoza samples [20, 46] from Uttarakhand.

CONCLUSION

It is clearly evident from the present study that the chilgoza pine nuts form a good substrate for *Aspergillus flavus* infestation and production of aflatoxins with potential hazard to the health of consumers. Although the quantities of chilgoza pine nuts consumed are in much lesser amounts posing any direct vulnerability, their applications in the preparation of many desserts and medicinal formulations with high incidence of *A. flavus* spores ardently require proper surveillance and monitoring.

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