



Mycobiota and aflatoxin (B₁ and B₂) contamination of apricot kernels (*Prunus armeniaca* L.) from Jammu and Kashmir, India

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Abstract

The aim of the present work was to assess the mycoflora and evaluate the aflatoxin contamination in apricot kernels from Jammu and Kashmir. A total of 83 samples collected from different regions of Jammu province were analysed for the mycoflora by different isolation techniques. Twenty nine fungal species belonging to 16 genera were recorded from these samples during mycoflora analysis. Species of *Aspergillus* were found to be the most frequent colonizers followed by the *Penicillium* among all the recorded moulds. In view of the predominance of *Aspergillus flavus*, a known producer of aflatoxins, screening of 83 contaminated samples was carried out for total aflatoxin levels using thin layer chromatography (TLC) and high performance liquid chromatography (HPLC). Out of these, 20 samples detected positive for aflatoxin B₁ and B₂ and the level of contamination ranged from 72.43-216.88µg/g and 0.11-235.32µg/g respectively which exceeds the tolerance limit of 20µg/g set by World Health Organization. The present study constitutes the first report on the incidence of mycoflora and aflatoxin contamination in dried apricot kernels from the markets of Jammu and Kashmir.

Keywords: Aflatoxin B₁ and B₂, Apricot kernels, *Aspergillus flavus*, HPLC, Mycoflora

Introduction

Aflatoxins (AFs) are the best known dietary human carcinogens of fungal origin and their occurrence in food commodities is a significant threat to public health. These are produced predominantly by two filamentous moulds, *Aspergillus flavus* and *Aspergillus parasiticus*. Besides, there are approximately twenty species of *Aspergillus* that have been known to produce about 20 structurally related aflatoxins; out of which B₁, B₂, G₁ and G₂ are of common occurrence with aflatoxin B₁ as the most toxic to human beings (Frisvad and Samson, 2004; Hedayati *et al.*, 2007; Rank *et al.*, 2011; Varga *et al.*, 2011; Zain, 2011; Soares *et al.*, 2012). The International Agency for Research and Cancer (IARC) has classified AFB₁ as a Group I hepatocarcinogen (IARC, 1993). Being chemically thermostable, aflatoxins are difficult to eliminate once produced (Risk Assessment Studies, 2001). Contamination of dried fruits and other stored edible commodities by various filamentous moulds and consequently the aflatoxin contamination have received much attention all across the globe (Zohri and Abdel Gawad, 1993; Rasheed *et al.*, 2004; Khosravi *et al.*, 2007; Lutfullah and Hussain, 2011;

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Rodrigues *et al.*, 2012; Ibrahim *et al.*, 2013). In India, surveys on the mould and mycotoxin contamination, particularly aflatoxins, have been taken on various fresh and dried fruits but a little interest has been paid towards dried apricot kernels that constitute one of the important indigenous dried edible products of Jammu and Kashmir, India that are among one of the world's best apricot kernels. Apricot kernels are derived from the hard seeds of apricot (*Prunus armeniaca*) belonging to family Rosaceae. This species grows in the temperate regions of Himachal Pradesh, Uttarakhand, Jammu and Kashmir and some parts of north-eastern states of India (Anonymous, 1969; Dixit *et al.*, 2010). Based on the taste, apricot kernels are of two types: bitter and sweet. Sweet kernels are widely used to add to flavour to desserts, puddings etc. and consumed as an important source of dietary protein, fat and fibre. In addition, its paste is used in baking cakes, pastries and coating chocolates. Oil extracted from the sweet kernel is either used in pure form or mixed with walnut oil whereas, oil extracted from bitter kernels are used in cosmetic industry and pharmaceutical preparations (Duke and Ayensu, 1985; Targais *et al.*, 2011). Seed kernels are also rich in iron, potassium, phosphorus, magnesium

and calcium with little proportion of sodium, copper, zinc and manganese. In addition, these seed kernels contain a high concentration of vitamin B₁₇, also called amygdalin, a nutrient that is believed to be beneficial in the treatment and prevention of cancer (Dwivedi *et al.*, 2007). In view of the adverse effects of mycobial spoilage and association of the aflatoxin production in various dried fruits, the present study was undertaken to evaluate the mycobial and natural aflatoxin contamination in dried samples of apricot kernels so as to assess the risk associated with the consumption of these kernels as well as their use in pharmaceutical preparations.

Material and Methods

(i) Isolation of mycoflora from dried apricot kernels and their identification

Eighty three samples of dried apricot kernels were randomly procured from the different retailers of Jammu province and Ladakh during April - December 2012. These samples obtained were collected in labeled sterilized polythene bags which were subsequently sealed and stored in a refrigerator at 4-5°C to avoid further contamination. Moisture content of the dried apricot kernels was determined by heating at 105°C for 2 hours to constant weight (AOAC, 1984). Mycological analysis was done by two methods. Standard blotter method as recommended by International Seed Testing Association (Gordan *et al.*, 1991) as well as dilution plating method following (Johnson and Curl, 1972) in a modified form using different media.

(a) Standard blotter method

In this method, all the samples were surface sterilised with 0.2% aqueous solution of sodium hypochlorite (NaOCl₂), rinsed twice with sterilised distilled water and were aseptically transferred to pre-sterilized wet blotting paper in sterilized Petri dishes, along with wet sterilized cotton swabs. After 4-5 days of incubation at 28±2°C, observations were made and the sporulating moulds were streaked on sterilized PDA supplemented with streptomycin sulphate (0.06g/l) to prevent the bacterial contamination. All the fungal species were isolated and purified by single spore isolation method. The pure fungal cultures recovered were grown on recommended sterilized Czapek yeast

autolysate agar medium (CYA), potato dextrose agar (PDA), Czapek-dox agar (CDA), potato sucrose agar (PSA), malt extract agar (MEA) media and identified on the basis of their cultural and micro-morphological characters using relevant literature.

(b) Dilution plating technique

In this technique 10g of the sample was taken in a 250ml Erlenmeyer flask containing 90ml sterilized distilled water and shaken thoroughly over mechanical shaker at constant speed for 30 minutes. The liquid was then decanted and centrifuged in sterilized centrifuge tubes at 3000rpm for 20 minutes. The residue, thus, obtained was mixed in 10ml distilled water and shaken vigorously to obtain a homogenous suspension. This suspension was aseptically spread plated on sterilized Petri dishes at the rate of 1ml per Petri dish by using a sterilized pipette. The two different media used for the isolation of the mycoflora were modified Czapek dox agar (CDA) and Dichloran rose bengal chloramphenicol agar (DRBC) medium supplemented with antibacterial agents streptomycin sulphate and chloramphenicol respectively. Five replicates per medium were maintained. The medium was poured by making gentle rotational movement of Petri dishes so as to ensure uniform spreading of the fungal spores and were incubated at 28±2°C for five days and percentage abundance of fungal species was calculated.

(ii) Evaluation of aflatoxins from naturally contaminated dried apricot kernels

Samples were analyzed for aflatoxin contamination by following the method of Thomas *et al.* (1975). A 50gm sub sample each of 83 finely ground dried samples was taken in 500ml Erlenmeyer flask containing 250ml of methanol and water (60:40v/v) and shaken on horizontal rotary shaker for 30 minutes. Thereafter, the mixture obtained was filtered and 125ml filtrate was taken in 250ml separating funnel along with 50ml n-hexane, shaken vigorously for 2 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 taken in a 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. For quantitative analysis, the processed sample was filtered with 0.45µm membrane filter and then transferred into HPLC vials for auto injection. All this processes were carried out in subdued light since aflatoxins are sensitive to light degradation.

(iii) Qualitative and Quantitative estimation of aflatoxins

Qualitative estimation of aflatoxins was done by thin layer chromatography (TLC). A 50µl aliquot of each sample extract was applied on activated TLC plates along with standard solutions, using a micropipette and then developed in a solvent system consisting of toluene: isoamyl alcohol: methanol (90:30:2v/v/v) in a sealed TLC tank. After developing, these plates were air dried and observed under long wave UV light (366nm). The chemical conformation was done by spraying 25% H₂SO₄ which changed the blue fluorescent spots to yellow as suggested by Stack and Pohland (1975). Aflatoxins were determined visually by comparing R_f 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 method described by Sigma-Aldrich (2000). Quantification was done by comparison of retention time (aflatoxin B₁- 6.02 minutes and B₂- 5.5 minutes) and peak areas observed in aflatoxin standards with those observed for the samples (Fig 1a).

(iv) Statistical analysis

All the experiments were performed in triplicate and data analysis was done on mean ± standard error subjected to one way analysis of variance (ANOVA). Differences among sample mean were reported to be significant when (p≤0.05). All statistical calculations were performed by using IBM SPSS 20.0 software.

Results and discussion

Twenty nine filamentous moulds belonging to 16 genera were recorded from 83 samples of the

visibly moulded apricot kernels during mycoflora analysis by employing dilution plating and standard blotter methods. Species of *Aspergillus* (24.13%) were found to be predominant among all the recorded moulds; *Aspergilli* including *Aspergillus flavus*, *A. fumigatus*, *A. japonicus*, *A. niger*, *A. sydowii*, *A. terreus* and *A. wentii* were the most frequent colonizers. These were followed by the *Penicillia* (10.34%) that included *Penicillium citrinum*, *P. oxalicum* and *P. purpurogenum* and *Fusarium* group (10.34%) was represented by *Fusarium oxysporum*, *F. solani* and *F. verticillioides* (Table 1).

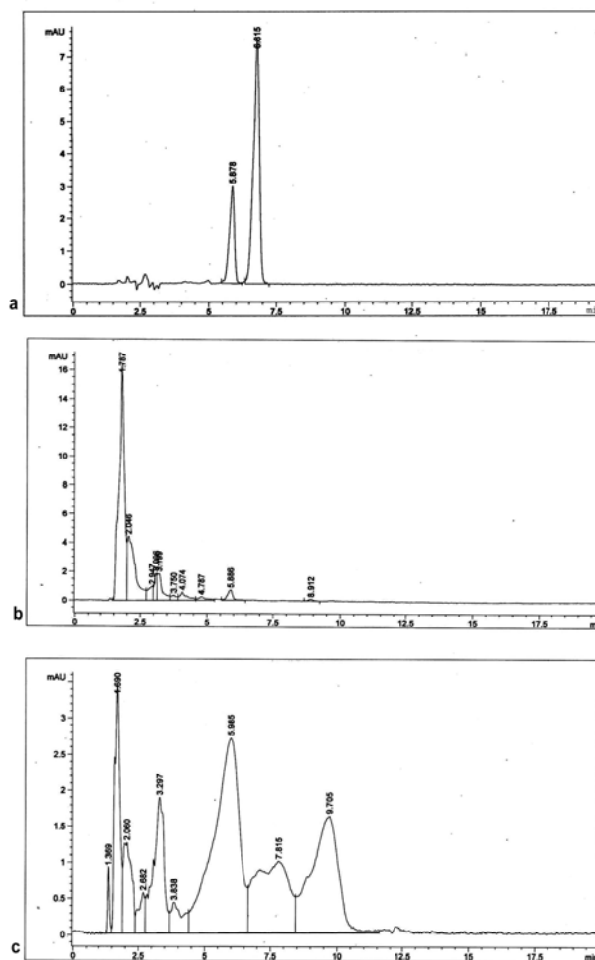


Fig 1. a) HPLC chromatograms of aflatoxin B₁ and B₂ standards b and c) aflatoxin B₂ from contaminated market samples of dried apricot kernels.

Previously, Weidonborner (2001) also reported the contamination of dried fruits with *Aspergillus*, *Penicillium* and *Fusarium* species. Dominance of

Table 1: Total mycoflora isolated from the dried apricot kernels along with their percentage abundance

Name of the fungal species	Jammu	Kathua	Poonch	Udhampur	Ladakh
<i>Acremonium roseum</i> ^{DPT}	0.20-6.15	-	2.40-12.40	-	-
<i>Alternaria alternata</i> *	0.40-9.75	20.2-23.85	3.09-8.18	7.73-25.50	-
<i>Aspergillus flavus</i> ^{DPT}	7.32-33.15	2.22-4.42	17.12-25.50	0.82-21.52	-
<i>A. fumigatus</i> ^{DPT}	0.82-1.46	-	3.20-6.10	-	-
<i>A. japonicus</i> ^{DPT}	0.33-7.73	0.82-2.4	-	-	-
<i>A. niger</i> *	1.20-5.62	-	4.33-12.51	-	-
<i>A. sydowii</i> *	2.40-9.40	10.12-25.68	-	2.0-23.65	0.63-1.09
<i>A. terreus</i> ^{DPT}	-	0.2-1.87	-	25.85-25.68	-
<i>A. wentii</i> *	1.20-5.89	0.12-2.0	2.34-12.51	-	-
<i>Cladosporium cladosporioides</i> ^{DPT}	2.02-6.40	4.62-19.49	-	11.92-19.22	-
<i>C. oxysporum</i> *	2.20-5.65	4.20-17.89	2.40-14.33	-	-
<i>Curvularia lunata</i> ^{SBM}	-	-	-	-	-
<i>C. pallescens</i> ^{DPT}	0.82-7.83	-	3.40-20.12	-	0.87-3.25
<i>Eurotium amstelodami</i> *	2.85-15.45	20.0-26.80	-	5.89-6.71	-
<i>Fusarium oxysporum</i> *	1.20-4.13	0.87-3.25	-	5.62-8.18	0.2-1.87
<i>F. solani</i> *	0.08-2.12	5.60-10.35	-	3.16-9.60	2.20-5.65
<i>F. verticillioides</i> ^{DPT}	0.87-14.55	2.40-6.82	-	-	-
<i>Geotrichum candidum</i> ^{DPT}	0.04-3.52	-	-	-	-
<i>Gliomastix murorum</i> ^{DPT}	0.62-2.09	-	-	5.09-17.89	-
<i>Mucor mucedo</i> *	0.20-2.79	12.20-24.68	5.40-13.73	1.46-8.36	-
<i>Paecilomyces lilacinus</i> ^{SBM}	-	-	-	7.83-15.45	-
<i>P. variotii</i> ^{DPT}	-	4.82-20.18	-	-	0.02-3.16
<i>Penicillium citrinum</i> *	-	3.20-8.20.18	3.82-16.60	-	-
<i>P. oxalicum</i> *	4.40-5.09	-	0.87-13.77	-	-
<i>P. purpurogenum</i> *	3.20-9.59	0.02-3.16	-	-	-
<i>Periconia saraswatipurensis</i> ^{DPT}	10.0-13.77	10.0-20.18	-	-	-
<i>Rhizopus oryzae</i> *	6.20-9.60	-	-	4.13-8.36	3.20-5.40
<i>Syncephalastrum racemosum</i> ^{SBM}	-	-	-	-	-
<i>Trichoderma harzianum</i> ^{DPT}	3.20-8.36	-	-	-	-
Total number of fungal species	23	17	11	12	6

DPT= Dilution plating technique SBM= Standard blotter method *= By both the methods

Aspergillus and *Penicillium* may be due to their ubiquitous nature and ability to grow on low moisture commodities. The high relative density of *Aspergilli* and *Penicillia* obtained during the present study supports the view of earlier workers (Abdel-Hafez and Saber, 1993; Hedayati *et al.*, 2010; Gupta *et al.*, 2013). The other monotypic filamentous fungi recorded were *Acremonium roseum*, *Alternaria alternata*, *Eurotium amstelodami*, *Geotrichum candidum*, *Gliomastix murorum*, *Mucor mucedo*, *Periconia saraswatipurensis*, *Rhizopus oryzae*, *Syncephalastrum racemosum* and *Trichoderma*

harzianum. Susceptibility of apricot kernels to a diverse group of fungal species suggests that the nutrient status of these kernels is adequate for the growth of these microfungi and unhygienic and unscientific pre- and post-harvest handling operations including storage, transportation, processing and marketing predispose them to fungal infestation. The incidence of mycoflora on apricot kernels reported in the present investigation concurs with the reports of Freire *et al.* (1999) who detected *Aspergillus flavus*, *A. niger*, *A. terreus*, *Eurotium amstelodami*, *Chaetomium globosum*, *Geotrichum candidum*, *Penicillium brevi*



compactum and *P. glabrum* while studying the mycobial contamination of cashew kernels collected from different markets of Brazil. Likewise, Goncalvez *et al.* (2008) isolated *Aspergillus flavus*, *A. niger*, *A. terreus*, *Fusarium* sp., *Penicillium* sp., *Phoma* sp. and *Rhizopus* sp. from Brazilian peanut kernels. From India, Sekar *et al.* (2008) isolated *A. flavus*, *A. niger*, *Mucor* sp., *Penicillium* sp. and *Rhizopus* sp. from dried fruits (figs, dates and grapes). Comparative analysis of mycoflora isolated from the market samples of apricot kernels procured from different districts showed considerable variation (Table 1). In general, the Jammu samples harboured highest fungal infestation containing 26 fungal species belonging to 16 genera followed by Kathua, Poonch, Udhampur and Ladakh. Overall, amongst the detected fungi from various districts, *Aspergillus flavus* was recovered with maximum percentage abundance followed by *Fusarium oxysporum*, *Eurotium amstelodami*, *Mucor mucedo*, *A. sydowii* and *Paecilomyces variotii*. Recovery of a large number of fungal species indicates that these kernels being rich in fats, protein and fibre provide ample nutrients to the surface invading fungi. Apricot kernels are almost sterile before the fruits are opened and seed kernels extracted, but since they are dehydrated under unhygienic environment by traditional sun-drying in open, they rapidly become colonized by the airborne fungal propagules. Moreover, environmental conditions and management practices during production, harvest, handling and storage affect the nature and degree of mycobiota contamination. The intrinsic moisture content, initial fungal inocula on the substrate and the storage system have been shown to affect the deterioration and spread of fungi during storage (Mittal and Mathur, 2003).

In the present study, the average moisture level of 12.5% was recorded in Jammu samples followed by the moisture level of 11.8%, 7.9 %, 8.3% and 7.5% in the samples obtained from Kathua, Poonch, Udhampur and Ladakh respectively. Increase in moisture level after drying in most of the stored seeds takes place because of their colloidal and hygroscopic nature due to which they absorb moisture from or give it up to the surrounding atmosphere until they are in equilibrium with it as observed by Pixton (1967) and Butt *et al.* (2004) in their studies on stored products. In addition, the

level of moisture permeability possessed by the packaging materials also influences the moisture content in storage (Kosoko *et al.*, 2009). In our study, a positive correlation was observed between the average number of fungal species recovered and percentage moisture content in these samples (Fig 2). In toxin evaluation studies, AFB₁ and AFB₂ were detected in 20 of 83 samples (24.09%).

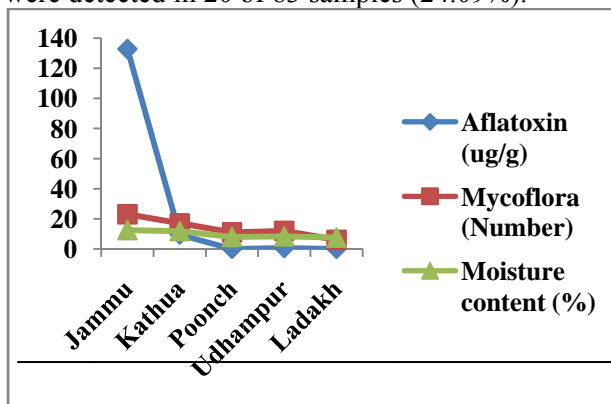


Fig2. Correlation among mycoflora, moisture content and aflatoxin contamination in dried apricot kernels

The incidence and level of aflatoxin contamination varied in the apricot kernel samples collected from various districts. Out of the five districts, the maximum number of positive samples for aflatoxin contamination was from Jammu (43.4%) followed by Kathua (40.0%) and Udhampur (26.6%). However, samples collected from Poonch and Ladakh districts were free from aflatoxin contamination. Samples collected from Jammu and Kathua were found to be highly contaminated with both aflatoxin B₁ and B₂ with detection levels ranging from 72.43-216.88µg/g and 4.97-235.32µg/g. From district Kathua only AFB₂ was detected having the concentration range from 0.19-39.8µg/g and one sample having both AFB₁ and AFB₂ (Table 2, Fig 1b, 1c). This higher incidence of aflatoxin contamination in samples obtained from Jammu and Kathua districts corroborates with the recovery of potential aflatoxin producing strains of *Aspergillus flavus* in higher percentage abundance from these samples as well as favourable agroclimatic conditions of these regions for the production of aflatoxins. The total amount and relative proportion of individual aflatoxin production and accumulation is influenced by the interaction of environmental factors such as temperature, moisture, relative humidity, water

activity and the prevalence of aflatoxin producing strains in the fungal flora and their relative toxigenicity (Bircan *et al.* 2008). The detection of high level of aflatoxins concurs with the findings of Luttfullah and Hussain (2011) and Imperato *et al.* (2011) who detected high contamination of aflatoxins at levels of 5.0-70.69 µg/kg in apricot kernels from Pakistan and Italy.

Table: 2 Incidence of natural aflatoxin contamination in the market samples of dried apricot kernels obtained from Jammu and Kashmir

Districts	No. of samples screened	Number of positive samples for aflatoxin	Number of positive samples			Range of aflatoxin B ₁ concentration (µg/g)	Range of aflatoxin B ₂ concentration (µg/g)
			B ₁	B ₂	B ₁ and B ₂		
Jammu	23	10 (43.4%)	2 (8.6%)	3 (13%)	5 (21.7%)	72.43-216.88 (132.88±26.22)*	4.97-235.32 (56.77±41.33)*
Kathua	15	6 (40%)	-	4 (26.6%)	2 (13.3%)	-	0.19-39.8 (9.48±9.20)*
Poonch	10	-	-	-	-	-	-
Udhampur	15	4 (26.6%)	-	4 (26.6%)	-	-	0.11-0.92 (0.60±0.24)*
Ladakh	20	-	-	-	-	-	-

Recently, Singh (2012) also reported low level of aflatoxin contamination in apricot kernel oil (0.2-4.0 µg/ml). Incidence of high level of aflatoxin contamination in other dried kernels, are also not uncommon from India (Sharma and Sumbali, 1999; Kishore *et al.*, 2002, Singh and Shukla, 2008; Sharma *et al.*, 2014). To the best of our knowledge, this is the first elaborative study on dried apricot kernels from different regions of the northern state of Jammu and Kashmir, India. The results of the present study showed that samples contained aflatoxins beyond the maximum tolerable limit (MTL) of 4 µg/kg set by European Union Commission for ready to eat dry fruits (EC, 2010). These levels of contamination also do not confer with the minimum permissible limits (MPL) of 20 µg/kg aflatoxins set forth by World Health Organisation and less than 30 µg/kg set by Indian health authorities (WHO, 1979; Sekar *et al.*, 2008). Obviously, it is a matter of serious concern, since the constant exposure to aflatoxins through contaminated products may have considerable impact on human health due to carcinogenic potential of these secondary metabolites (William *et al.*, 2004).

Conclusions

The present study demonstrated that there is a need for implementation and optimization of pre-harvest handling operations, sanitized extraction of apricot kernels and their mechanical drying to reduce the

moisture content to the safer level for storage. Moreover, the high incidence of aflatoxins emphasizes the need for regular monitoring and a more stringent food safety system in order to control the aflatoxins at the lowest possible level. This would enhance purity and security in dried kernels and provide great protection to the health of consumers as they are used in preparing various desserts, cosmetics and pharmaceutical preparations destined for direct human consumption. Also, there is an urgent need to design a good means of reducing this contamination so as to meet the international standard of good manufacturing practice.

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