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Mycoflora and natural aflatoxin contamination in dried quince seeds from Jammu, India

Pinky Bala, Dimple Gupta and Y.P. Sharma*
Department of Botany, University of Jammu, Jammu - 180 006, India
*Corresponding Author E-mail: **yashdbm3@yahoo.co.in**

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Abstract

Eighty two samples of dried quince seeds, obtained from the markets of Jammu province, were examined for mycoflora by different isolation techniques. A total of 27 fungal species belonging to 11 genera were recovered and identified from these samples. The predominant fungal genera encountered were *Aspergillus*, *Penicillium* and *Fusarium*. In view of the predominance of Aspergillus flavus, a known producer of aflatoxins, screening of the fungal contaminated samples was carried out for total aflatoxin levels using high performance liquid chromatography (HPLC). Twenty one aflatoxin positive samples contained 8.07-33.45 µg g^{-1} and 0.05-3946.97 µg g^{-1} AFB1 and AFB2 respectively. These results suggest that biochemical composition of dried quince seeds, along with climatic conditions of the region seem to be very favourable for aflatoxin production by toxigenic strains of *A. flavus*. Therefore, monitoring of aflatoxins in dried quince seeds is recommended for this region.

Key words

Aflatoxin, *Aspergillus*, HPLC, Quince seed

Introduction

Aflatoxins (AFs) mainly produced by toxigenic strains of *Aspergillus flavus* and *A. parasiticus* have received significant attention throughout the world because of their hepatocarcinogenic, teratogenic and immunosuppressive properties (Leontopoulos *et al*., 2003; Paterson, 2007). About 4.5 billion people in developing countries are chronically exposed to uncontrolled amount of aflatoxin which results in changes in nutrition and immunity (Williams *et al*., 2004). They are extremely durable under moist conditions of storage, handling and processing of foods. Among 18 different types of aflatoxins identified, the major AFs are B_1, B_2, G_1 and G_2 (based on their fluorescence under UV light: blue or green) (Iqbal *et al*., 2010; Zain, 2010), and AFB_1 is considered as the most carcinogenic compound by the International Agency for Research as Cancer (IARC, 1982). Due to high level of toxigenicity, aflatoxin contamination in edible commodities has attracted attention of several workers (Freire *et al*., 1999; Sharma and Sumbali, 1999; Iqbal *et al*., 2010; Gupta *et al*., 2013; Sharma *et al*., 2013; Bala *et al*., 2014; Yogendrarajah *et al*., 2014; Azaiez *et al*., 2015).

Quince seeds, commonly called as 'Bihidana', are obtained from the fruit of *Cydonia oblonga* Mill. and are indigenously grown in Jammu and Kashmir and hilly tracts of Himachal Pradesh. Seeds extracted from ripened fruits are known to possess various medicinal properties and find use in cosmetic industry (Elandsen and Magney, 1992). They are rich in phenolics, mucilage, fatty acids and free amino acids (Silva *et al*., 2005). In addition, they are also known to contain fat soluble bioactive compounds such as C-glycosyl flavones, tocopherols and phytosterols that are known to reduce low density protein (LDL) cholesterol level. Fruit and seeds together are used for de-addiction (antialcohlic), carminative, expectorant, antibacterial, conjunctivitis, cough, bronchitis, constipation, diarrhoea, stomach, ulcers, healing on skin lesions etc. (Velickovic *et al*., 2001; Rodriguez *et al*., 2009; Sharma *et al*., 2011). Inspite of using all the available means of food protection, spoilage of edible commodities is still a major problem in different parts of the

world. High temperature, high rainfall and relative humidity in these growing areas are highly conducive for proliferation of mycoflora and aflatoxin production. Apart from the climatic conditions, lack of Good Agricultural Practices (GAP) and Good Manufacturing Practices (GMP) are of great concern in developing states where these quinces are grown. *Aspergillus*, *Penicillium* and *Fusarium* pose serious mycotoxicological risks in variety of food products as they can produce several mycotoxins causing serious problems for animal and human health worldwide. Seeds are not exceptional in this regard as they may be exposed to a wide range of microbial contamination from farm- to- fork due to poor collection conditions, unpretentious production process and extended drying times. The traditional method of drying quince seeds in open air under the sun, is still a common practice in Jammu, which potentially exposes them to the risk of contamination. However, the presence of mycoflora and mycotoxin contamination in quince seeds is meagre therefore the present study was carried out to identify mycoflora and aflatoxin contamination in dried quince seeds that are commonly used in the manufacture of various cosmetic and commercial drugs and can be toxic to human health.

Materials and Methods

Collection of samples : A total of 82 samples of dried quince seeds were randomly procured from different retailers of six districts grown indigenously in Jammu, Kathua, Poonch, Rajouri, Udhampur and Doda of Jammu region during January - December, 2013. These samples were labeled, packaged in sterile polyethylene bags, transferred to the laboratory and kept in a cool place (3-5°C) till fungal isolation, identification and aflatoxins analysis.

Moisture content : The method of Mandeel (2005) with some modifications was used for determining the moisture content. Known weight of samples were dried at 100ºC for 4 hrs and their difference in weight was calculated.

$$MC = [(Wi-Wf) / Wi] \times 100$$

Where, MC= moisture content; Wi = Initial weight and Wf = final weight

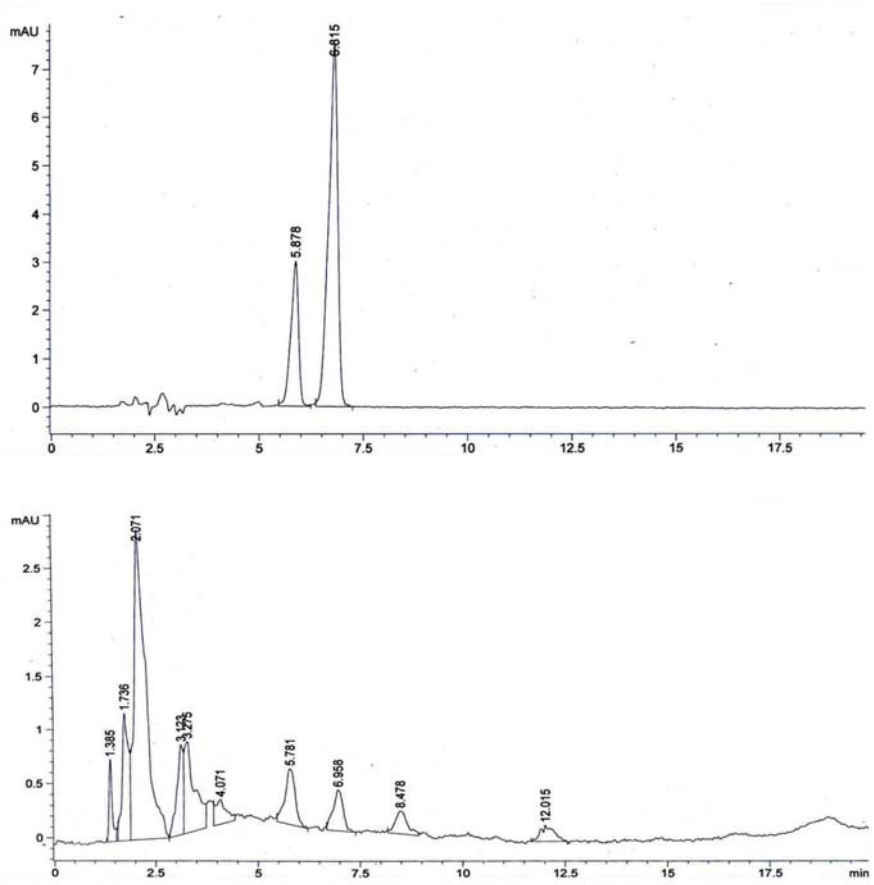

Fig. 1 : HPLC chromatograms of (a) aflatoxin B1 and B2 standards (b) aflatoxin B1 and B2 from contaminated market samples of dried quince seeds

Mycoflora analysis : Mycological analysis was carried out by two methods *viz*., standard blotter method as recommended by International Seed Testing Association ISTA (1966) and dilution plating by following the method of Harrigan (1998) with some modifications.

Screening of toxigenic strains of *Aspergillus flavus* recovered from market samples of dried quince seeds : Aflatoxin producing potential of *Aspergillus flavus* isolates from dried market samples of quince seeds was tested in rice flour liquid medium (Misra and Sinha, 1979). Erlenmeyer flasks containing 100ml of autoclaved rice flour medium were inoculated with 1ml of spore suspension (~10^6 spores per ml) from 7-day-old culture 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 and 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 darkness for qualitative and quantitative analysis.

Aflatoxin analysis : Aflatoxins were extracted from dried quince seeds according to the method described by Schuller *et al*. (1983). 50 g of each ground sample was added to 500 ml conical flask containing 50ml distilled water and 100 ml chloroform. The flasks were shaken for 30 min on a rotator shaker and the suspensions were filtered. The resulting chloroform extracts were purified following the method of Takeda *et al*. (1979) with some modifications. Elutes were evaporated to dryness on steam bath. Each residue was re-dissolved in 1ml chloroform. Analysis was performed using HPLC (ABILENT) consisting of liquid chromatographic pump LC-10 AT, an auto injection system SIL-10 A with 50µl sample loop, a variable wavelength absorbance detector SPD-10, reverse phase analytical column PSEM-SPADION (250 × 4mm) filled with ODS(M), RP-18 material, 5µm particle size (Merck). Isocratic elution was done with water: acetonitrile: methanol (54:34:12v/v/v) at a flow rate of 1ml min^{-1}. Injection volume of extract solution was 30µl. A variable length UV-VIS detector set at 365 nm was used. All the analysis was performed at room temperature and recorded in HP Deskjet 670C. Aflatoxins in the samples was detected by comparing the retention time (aflatoxin B_1- 6.02 minutes

Table 1 : Total mycoflora isolated from dried quince seeds along with their percentage abundance

Fungal species	Jammu	Kathua	Poonch	Rajouri	Udhampur	Doda
Acremonium roseum DPT	0.02 - 4.72	9.22-16..21	12.71-22.80	0.63-1.09	2.40-12.40	-
A. strictum *	-	-	-	-	3.09-8.18	-
Alternaria alternata *	5.40-13.85	7.42-10.34	-	-	17.12-25.50	-
Aspergillus ficcum SBM	-	-	4.42-10.58	-	3.20-6.10	-
A. flavus *	22.50-42.80	15.17-22.50	6.62-23.12	8.23-20.2	6.31-12.22	4.23-9.22
A. fumigatus *	-	3.32-4.19	3.34-6.19	3.20-5.24	-	-
A. japonicus *	0.06-2.15	-	-	-	-	-
A. niger *	0.08-2.15	8.08-20.12	4.32-9.75	-	4.33-12.51	0.02-3.16
A. sydowii DPT	5.65-20.13	-	6.60-15.82	0.87-3.25	-	-
A. terreus *	2.24-6.35	0.42-5.22	-	-	-	-
*A. oryzae**	2.23-7.14	-	2.21-8.98	3.12-5.54	-	-
A. wentii DPT	-	0.88-13.15	2.24-5.65	0.2-1.87	2.34-12.51	-
Cladosporium cladosporioides *	2.38-11.12	1.51-25.26	4.20-20.13	2.20-5.65	-	10.0-19.18
C. oxysporum DPT	6.61-11.38	0.34-2.38	-	-	2.40-14.33	4.82-13.77
Drechslera australiensis *	2.24-6.82	-	0.88-6.61	-	-	-
Fusarium pallidoroseum *	-	-	3.21-23.03	-	3.40-20.12	-
F. solani DPT	0.88-14.51	0.04-3.21	-	-	-	-
F. verticillioides *	-	5.40-9.37	0.88-9.22	-	-	-
Gliomastix murorum *	-	12.12-23.24	-	0.02-3.16	-	-
Mucor mucedo *	1.51-12.82	-	-	-	-	-
Paecilomyces lilacinus DPT	3.21-15.84	-	2.20-5.15	-	-	3.20-9.59
P. variotii *	-	0.04-1.51	-	-	-	2.40-6.82
Penicillium citrinum *	3.12-12.10	2.88-10.87	-	-	5.40-13.73	-
P. oxalicum DPT	-	3.15-5.15	-	3.20-5.40	-	-
P.purpurogenum DPT	-	-	-	0.83-2.12	-	-
Penicillium madriti DPT	5.12-12.60	-	2.51-13.04	-	-	-
Rhizopus oryzae *	9.22-13.31	0.02-3.12	12.51-14.25	-	3.82-16.60	-
Total number of fungal species	**17**	**16**	**15**	**10**	**11**	**6**

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

and B_2- 5.5 minutes) and peaks generated with that of standards (Fig. 1).

Statistical analysis : Statistical significance of the experiment was analysed by ANOVA and presented at $p \leq 0.05$. All the statistical calculations were performed by IBM SPSS 20.0 software.

Results and Discussion

Mycological analysis : In the present study, eighty two dried quince seed samples collected from Jammu markets were investigated for fungal contamination. A total of twenty seven fungal species belonging to eleven genera were isolated by dilution plating technique and standard blotter method (Table1). *Aspergillus* (33.3%) was the most predominant genus in contaminated samples represented by *Aspergillus ficcum*, *A. flavus*, *A. fumigatus*, *A. japonicus*, *A. niger*, *A. oryzae*, *A. sydowii*, *A. terreus* and *A. wentii*. Other fungi included of *Penicillium* group including *Penicillium citrinum*, *P. oxalicum*, *P. madriti* and *P. purpurogenum* and the *Fusarium* group (*Fusarium pallidoroseum*, *F. solani* and *F. verticillioides)*. Two species each of *Acremonium* (*A. strictum* and *A. roseum*), *Cladosporium* (*C. cladosporioides* and *C. oxysporum*), *Paecilomyces* (*P. liliacnus* and *P. variotii*) were also detected. The other monotypic filamentous fungi were *Alternaria alternata*, *Drechslera australiensis*, *Gliomastix murorum*, *Mucor mucedo* and *Rhizopus oryzae*.

The incidence of mycoflora on dried quince seeds reported in the present investigation concurs with the reports of Misra (1981) and Roy *et al*. (1988) who detected *Aspergillus flavus*, *A. niger*, *A. fumigatus*, *A. ochraceus*, *A. candidus*, *A. sydowii*, *Chaetomium dolicholricum*, *Fusarium moniliforme*, *Penicillium oxalicum*, *Alternaria* sp., *Curvularia* sp. and *Rhizopus* sp. from the seeds of *Amomum subulatum*, *Coriandrum sativum*, *Cuminum cyminum*, *Foeniculum vulgare*, *Piper nigrum* and *Cinnamomum zeylanicum*. Similarly, Bankole *et al*. (1999) isolated *Alternaria* sp., *Botrydioploidia theobromae*, *Cladosporium* sp., *Fusarium* sp., *Macrophomina phaseolina*, *Aspergillus* sp., *Penicillium* sp. and *Rhizopus* sp. from stored melon seeds of Nigeria. In *Zanthoxylum armatum*, Gupta *et al*. (2013) isolated 18 fungal species; the most commonly occurring species were *Aspergillus flavus*, *A. glaucus*, *A. niger*, *A. ochraceus*, *A. oryzae*, *A. versicolor*, *Mucor mucedo*, *Paecilomyces lilacinus*, *P. victoriae* and *Penicillium chrysogenum*. Recently, El-Wakil (2014) isolated *Aspergillus flavus*, *A. niger*, *Alternaria alternata*, *Curvularia lunata*, *Fusarium moniliforme*, *F. oxysporum*, *F. semitectum*, *Penicillium digitatum*, *Stemphylium* sp. and *Trichoderma* sp. from sun flower seeds collected from different locations of Egypt.

The existence of diversity of filamentous fungi on these dried quince seeds during storage and marketing is also partially consistent with the reports of various other researchers (Alghalibi and Shater, 2004; Kumar *et al*., 2011; Venugopalan and Giridhar, 2012; Toma and Abdullah, 2013). In general, the samples of Jammu harboured highest fungal infestation containing 17 fungal species belonging to 10 genera followed by Kathua, Poonch, Udhampur, Rajouri and Doda. Hot and humid conditions prevailing in Jammu region might be the factor responsible for their maximum deterioration. Overall, amongst the detected fungi from various districts, *Aspergillus flavus* was recovered with maximum percentage abundance followed by *Alternaria alternata*, *Cladosporium cladosporioides*, *Gliomastix murorum* and *Fusarium pallidoroseum.*

Detection of diverse group of fungi indicates heavy mycobial load on these market samples destined for human consumption. Quince seeds are almost sterile before opening of fruits and extraction of seeds and therefore, these species might be associated with these samples during unhygienic and unscientific pre- and post-harvest handling operations. Once colonized by mycoflora, the substrate composition of quince seeds having mucilage and fatty oils favours growth

Table 2 : Incidence of natural aflatoxin contamination in the market samples of dried quince seeds obtained from various districts of Jammu province

Districts	Number of samples screened	Number of positive samples for aflatoxin	Number of positive samples B_1	B_2	B_1 and B_2	Range of aflatoxin B_1 concentration ($\mu g\, g^{-1}$)	Range of aflatoxin B_2 concentration ($\mu g\, g^{-1}$)
Jammu	18	8 (44.4%)	-	3 (16.6%)	5 (27.7%)	8.07-33.45 **(19.20 ± 4.56)***	57.91-3946.97 **(882.90 ± 766.30)***
Kathua	19	5 (26.3%)	-	3 (15.7%)	2 (10.5%)	9.27-15.78 **(12.525 ± 2.05)***	1.13-107.42 **(22.28 ± 21.28)***
Poonch	18	3(16.6%)	-	3 (16.6%)	-	-	0.05-0.43**(0.2415 ± 0.15)***
Rajouri	10	2 (20.0%)	-	2(20.0%)	-	-	4.97-235.32 **(56.77±41.33)***
Udhampur	10	3 (30.0%)	-	3 (30.0%)	-	-	4.41-52.38**(21.67 ± 15.38)***
Doda	7	-	-	-	-	-	-

*mean±SE

and proliferation of these contaminants. Moisture content of seeds during storage is one of the factors determining the kind and quality of mycoflora on seeds, which in turn judges the nutritive value (Singh *et al*., 2008). In quince seeds, average moisture level of 16.7% was recorded in Jammu samples followed by Kathua (15.1%), Poonch (13.3%), Udhampur (12.7%) Rajouri (8.9%) and Doda (7.9%). High moisture content of seeds resulted in infestation by fungi more so with *Aspergillus* sp. Among the *Aspergillus* sp., *Aspergillus flavus*, *A. fumigatus, A. niger* and *A. sydowii* were predominant at higher and lower moisture levels, respectively.

Thirty eight isolates of *A. flavus* were obtained from 82 samples of dried quince seeds. Of these, 11 (28.9%) isolates were found to be toxigenic with blue spots on TLC plates. Analysis of aflatoxigenic potential of *A. flavus* isolates showed production of AFB_1 and AFB_2, with levels ranging from 12.32-241.291 and 8.231-149.103µg g^{-1} respectively. Comparatively, the amount of aflatoxin B_1 produced by these toxigenic isolates was higher than aflatoxin B_2. Seven isolates showed the presence of both these toxins where as three isolates screened positive for AFB_2 only.

The study on the occurrence of AFs contamination in dried quince seeds revealed high percentage of AFB_1 and AFB_2 in samples ranging from 8.07-33.45µg g^{-1} and 0.05-3946.97µg g^{-1} respectively. In the present study, co-occurrence of both AFB_1 and AFB_2 was also observed. Among the positive samples, 8.53% were found to be co-contaminated with both toxins. Different concentrations of both the groups of aflatoxins were detected from the samples collected from different districts. Highest aflatoxin contamination was observed in the samples procured from markets of Jammu district (44.4%) followed by Udhampur (30.0%), Kathua (26.3%), Rajouri (20.0%) and Poonch (16.6%). However, samples collected from Doda district were free from aflatoxin contamination. Samples collected from Jammu and Kathua were found to be highly contaminated with both aflatoxin B_1 and B_2 with detection levels ranging from 8.07-33.45µg g^{-1} and 1.13-3946.97µg g^{-1}. Least aflatoxin contamination was detected in samples collected from Poonch district where in only 2 samples were found contaminated with AFB_2 having a mean range of 0.05-0.43µg g^{-1}. The results showed that all the positive samples of dried quince seeds contained aflatoxins beyond maximum tolerable limit of 4µg kg^{-1} set by the European Union Commission for ready to eat dry fruits (EC, 2010). These levels of contamination also do not confer with the minimum permissible limits of 20µg kg^{-1} aflatoxins set forth by World Health Organisation and less than 30µg kg^{-1} set by the Indian health authorities (WHO, 1979; Sekar *et al*., 2008). High level of aflatoxin contamination in these seeds can be explained on ubiquitous prevalence of toxigenic *A. flavus* as a natural contaminant in dried samples. The processing steps involved during harvesting and drying and relative humidity ≥ 90 and temperature at 30ºC are suitable conditions for attack of *Aspergillus flavus* in dried commodities (Horn, 2003; Klich, 2007).

So far, there has been no report published on mycoflora and aflatoxin contamination in dried quince seeds. However, detection of high level of aflatoxins concurs with the findings of Elshafie *et al*. (2002) who detected high contamination of aflatoxins at a mean level of 50µg l^{-1} to 90 µg l^{-1} in different medicinal spices purchased from five popular companies in the Sultanate of Oman. In an another study from Iran, Behesti and Asadi (2013) also reported low level of aflatoxin contamination in sunflower seeds at a mean level of 40.68ng g^{-1} and a mean level of 2.81±0.44ng g^{-1} in safflower seeds. A recent investigation of AFs in dates and dried fruits from Tunisian and Spanish markets, Azaiez *et al*. (2015) reported incidence of these toxins at a mean level of 1.1-16.5µg kg^{-1}.

From the results of the present investigation, it can be concluded that high level of aflatoxin contamination in dried quince exceeding the permissible limits set forth by Indian authorities and also recovery of aflatoxigenic *Aspergillus flavus* isolates from this dried commodity of medicinal importance is a matter of serious concern as these seeds are used as an important ingredient in various herbal formulations. Since consumption of these herbal drugs have shown an upbeat trend in recent times, it is imperative to scrutinize dried quince seeds and their products for aflatoxin contamination for quality evaluation and formulating management strategies.

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