RESEARCH ARTICLE

SURVEY OF MYCOFLORA AND MYCOTOXIN CONTAMINATION IN DRIED DATE PLUM PERSIMMONS (*DIOSPYROS LOTUS* L.) FROM MARKETS OF JAMMU PROVINCE

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Abstract: The present investigation was designed to analyze moulds and mycotoxins responsible for biodeterioration of the market samples of dried fruits of date plum persimmon (*Diospyros lotus* L.). A total of 31 fungal species were isolated from 65 samples by two different isolation techniques. Assessment of mycobial load exhibited the presence of many such fungal species that are broadly acknowledged as the most important mycotoxin producers. Mycotoxins were assessed by high performance liquid chromatography (HPLC). The mycotoxins detected were aflatoxins, ochratoxin A and patulin. Out of 65 samples, 69.23% of dried date plum persimmon samples were found contaminated with aflatoxins in the range of 0.78-798.34µg/kg and 47.69% samples have ochratoxin A (OTA) contamination (0.76±432.09µg/kg). Patulin was quantified in 32.30% samples in the range of 0.89-123.90µg/kg. The present study constitutes the first report of fungal and mycotoxin contamination in dried date plum persimmons from India.

Keyword: Mycoflora, Mycotoxin, *Diospyros lotus*, Survey

INTRODUCTION

India is the only country in the world where due to existence of variable agroclimatic conditions every kind of fruit is grown and is the second largest producer of fruits with an annual production of 88.8 million tonnes from an area of 6.3 million hectares (Anonymous, 2015-16). Fruits constitute commercially and nutritionally important indispensable food commodity. It plays a significant role in the diet of many people by raising its nutritional value by providing essential minerals, vitamins, proteins, calorie contents and addition of flavours, colours and variety to the diet (Aluko, 1989; Okigbo, 2000; Al-Hindi *et al*., 2011).

Fruits are highly perishable due to their high moisture andnutrientsand thus become prone to injury and subsequent attack by a diversity of microorganisms. The drying of fruits concedes for their better preservation by reducing water content, consequently restraining microbial growth and enzymatic modifications. Preservation of fruits by solar drying has been practiced for centuries. However, products derived from traditional sun drying usually exhibit poor quality due to unhygienic storage conditions. During drying, air borne filamentous fungi may contaminate fruits and causes undesirable losses in dry matter by utilizing carbohydrates, degrading lipids and proteins as energy source and secrete potential mycotoxins into them. The presence of these biological contaminants has varying effects on the health and productivity of humans (Shephard, 2008).

Presently, among all food contaminants (synthetic contaminants, food additives or pesticide residues), mycotoxins are considered as the most important chronic dietary risk factor, demonstrated from the constant international attention paid due to their noxious impact on health and economy of several countries, particularly the developing ones (Van Egmond *et al*., 2007). Mycotoxins can occur both in temperate and tropical regions of the world, depending on the producing mould species. Major food commodities affected are cereals, nuts, dried fruits, coffee, cocoa, spices, oil seeds, dried peas and beans (Turner *et al*., 2009). Different surveys and monitoring programmes have been carried out in several countries attempting to obtain a overall pattern of mycotoxin contamination in dried fruits (Zorzete *et al*., 2013; Essawet *et al*., 2017; Gupta *et al*., 2017; Skrbic *et al*., 2017, Wei *et al*., 2017).

More than 400 different mycotoxins are known.Among these secondary metabolites, aflatoxins (AFs), ochratoxin A (OTA) and patulin (PAT) toxins are the most toxic and widely distributed mycotoxins in dried fruits. Aflatoxin are difuranocoumarin derivatives formed by a polyketide pathway by fungi that belong to *Aspergillus* genus and particularly by *A. flavus*, *A. parasiticus* and rarely by *A. nomius* (Cary and Ehrlich, 2006; O' Riordan and Wilkinson, 2008). Owing to hydrothermal conditions that facilitate their production, aflatoxins are generally regarded as main contaminants usually in geographical areas exhibiting tropical or sub-tropical climate (Cotty and Garcia, 2007). Ochratoxin A (OTA), a well characterized nephrotoxin, is mainly produced by *Aspergilluscarbonarius*, *A.niger* and *A.ochraceus* in tropical climates and by *Penicilliumverrucosum* and *P.nordicum* in temperate zones (Richard, 2007; Turner *et al*., 2009). On a global scale, the contamination of spices, dried fruits and nuts by

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ochratoxin A was reported by several researchers (Bircan, 2009; Rahimi and Shakerian, 2013; Bala *et al*., 2016; Bala *et al*., 2017; Gupta *et al*., 2017; Heshmati *et al*., 2017). Another common toxic metabolite Patulin (PAT) is produced by a variety of moulds, particularly *Aspergillus*, *Penicillium* and *Byssochlamys* (Pitt and Hocking, 1999; Fung and Clark, 2004). Patulin exhibits mutagenic and carcinogenic properties in several animal species and induces intestinal injuries, including epithelial cell degeneration, inflammation, ulceration, and haemorrhage (Mahfoud *et al*., 2002).

Date plum persimmon (*Diospyroslotus* L.; family Ebenaceae), a deciduous tree, is native to China and Asia. Locally known as amluk in Hindi and is cultivated in several countries for its edible fruits. The fruits are globose, 1.5-2.0cm in diameter and bluish-black in colour when ripe (Davis, 1978). The mature fruits of persimmon are normally not consumed directly after harvesting because of their astringent taste, but are dried and then sold in the markets (Ayaz and Kadioglu, 1999). In traditional Chinese medicine, the fruits are febrifuge and frequently used to stimulate secretions and seed as a supplement for being sedative (Gao *et al*., 2014). Fruits contain sugar, fatty acid (palmitic acid, palmitoleic acid, stearic acid, linolenic acid, oleic acid etc. (Glew *et al*., 2005). Nevertheless, consumption of the contaminated dried fruits are pernicious to human health and also resulted in economic losses. Hence, the present investigation aims in determining the mycoflora contaminating dried date plum persimmons sold in markets of Jammu and assessing the natural incidence of mycotoxin in them.

MATERIAL AND METHOD

Sample collection

A total of sixty five dried date plum persimmons samples were collected randomly from different regions of Jammu province including Jammu, Udhampur, Rajouri and Poonch during January 2012- March 2014. These sites were chosen on the basis of their availability in the market. These samples were usually found outside, kept in metal or plastic containers, wooden boxes or gunny bags. Care was also taken to avoid old stocks and visibly contaminated samples. The samples were collected in sterilized polythene bags, sealed over the flame to prevent moisture changes andstored at low temperature (5ºC) for further mycobial assay.

Mycological analysis

Standard blotter method and serial dilution method were employed to isolate the mycoflora associated with dried date plum persimmons species. Experiment was performed in replicates for both the methods.

Standard blotter method. All the 65 dried fruit samples were surface sterilized with 0.1% sodium hypochlorite, rinsed twice with sterilized distilled water and were aseptically transferred to pre-sterilized wet blotting paper in sterilized Petri plates, along with wet sterilized cotton swabs. After 3 days of incubation at 28 ± 2^0C, observations were made and the sporulating moulds were streaked on PDA plates.

Serial dilution method The moulds in dried fruits were isolated following the method of Gnonlonfin *et al*. (2008) with some modifications. A 10g sample was taken in 250ml Erlenmeyer flask containing 90ml sterilized distilled water and shaken thoroughly on a mechanical shaker at 200 rpm for 30 minutes. Dilutions of residue were made with sterilized distilled water. 1ml aliquot of each dilution were then transferred to Petri plate containing two different isolation media- dichloran rose bengal chloramphenicol agar medium (DRBC) and malt salt agar (MSA). Five replica plates per medium were used and were incubated at 28 ± 2^0C for 5 days. The results were expressed as average colony forming units per gram of sample (cfu/g) using the following formula (Jakovac-Strajn *et al*., 2010):

Average colony forming units per gram of sample (cfu/g)

$$N= \Sigma C/ Vxnxd$$

N = Number of colony forming units per gram of sample (cfu/g)

$\sum C$ = Sum of all colonies of the count

V = Volume of the dilution pipette in the count plates in ml

n = Number of count plates that can be evaluated

d = Dilution factor

Percentage frequency of each fungal species was calculated by using the formula given

$$\text{Frequency (\%)} = \frac{\text{Number of samples from which an organism was recovered}}{\text{Total number of samples tested}} \times 100$$

Identification and maintenance of fungal cultures. Fungal species recovered were grown on sterilized recommended media and identified on the basis of their cultural and micro-morphological characters using relevant literature and recommended keys (Gilman 1957; Raper and Fennell 1965; Ellis 1971; Pitt 1979; Pitt and Hocking 2009).

Extraction of mycotoxins from naturally contaminated dried date plum persimmons

Modified multimycotoxin method developed by Roberts and Patterson (1975) was employed for detection and estimation of mycotoxins in naturally contaminated market samples. The dried samples were finely crushed in an electric grinder. 25g of crushed sample was taken in 250ml Erlenmeyer flask containing 100ml mixture of acetonitrile and 4% potassium chloride (90:10v/v) and shaken on horizontal rotary shaker for 30 minutes. Thereafter, the mixture was filtered and filtrate was defatted thrice with 50ml isooctane in separating funnel (250ml capacity). Upper lipid containing layer was

discarded and added 12.5ml distilled water was added to lower acetonitrile layer. Mycotoxins were extracted from lower layer by using 20ml chloroform. Lower chloroform layer was collected in a conical flask and filtered through Whatman no. 42 filter paper having a bed of anhydrous sodium sulphate. The extract was collected in a beaker and then evaporated to dryness on a water bath. The residue was dissolved in 1ml of benzene: acetonitrile (98:2v/v) solution and stored in a clean screw cap vial at -20°C in the darkness for quantitative detection of mycotoxins.

Quantitative estimation of mycotoxins

Chromatographic separations were performed on a HPLC system, SHIMADZU consisting of a binary pump (LC-20AD), equipped with DGU-20A5R microvacuum degasser, a thermostated autosampler (SIL-20ACHT), a column oven (CTO-ASVP), a photodiode array UV-VIS detector set at different wavelengths (Table 1). The analytical component was C-18G (250 x 4.6mm). The mobile phase consisted of different solvent systems for different mycotoxins (Table 1). Analysis was performed at room temperature (25-30^{0}C) and data was recorded in HP Compaq.

Statistical Analysis

All the experiments were replicated thrice and data sets were statistically analyzed using IBM SPSS 20 software. The statistical level of significance was fixed at $P<0.05$.

RESULT AND DISCUSSION

Moulds, as usual dwellers of soil and contaminants of air, water, food and feed, can be found predominant all over the globe. Factors such as temperature, pH, moisture content, oxygen level, nutritional components and microbial competition influence mould growth. They are the momentous destroyers of numerous edibles during storage and render them unfit for human consumption by retarding their nutritive value. As depicted in table 2, dried date-plum persimmons harboured 31 fungal species belonging to 9 genera representing strong generic and specific variability of fungal organism on this substrate. The genus *Aspergillus* was the most frequent colonizer that comprised of fourteen species and thus formed the largest diversity among all the recovered genera. Penicillia group accounted for eight species *viz*., *P. aurantiogriseum*, *P. chrysogenum*, *P. citrinum*, *P. corylophilum*, *P. expansum*, *P. fellutanum*, *P. purpurogenum* and *P. verrucosum*. These storage moulds are capable of flourishing well at low moisture substrates with minimum or no competition from other fungi (Srivastava *et al*., 2011). The high prevalence of *Aspergillus* and *Penicillium*species in the dried fruits supports the view of earlier mycologists (Alghalibi and Shater, 2004; Toma and Rajab, 2014; Saadullah and Abdullah, 2015; Tournas *et al*., 2015). Genus *Eurotium* and *Scopulariopsis* were represented by two species each (*Eurotiumchevalieri*, *E. rubrum*,*Scopulariopsisbrevicaulis* and *S. brumptii*. Other filamentous fungi were *Alternaria alternata*,*Curvularia lunata*, *Mucor mucedo*, *Rhizopus stolonifer* and *Syncephalastrum racemosum.*

The growth and proliferation of moulds is also strongly related to the seasons and geographical conditions of the area. Comparative mycological survey of the samples collected from local markets of Jammu province showed various types of fungal species with varying degree of frequency of occurrence. Perusal of data in table 2 depicted that the maximum numbers of fungal species (21 species) were recovered in the range of $0.5x10^3$-$8.3\ x10^3$cfu/g from samples procured from Jammu district. Among the recovered fungal species, Zygomycota showed 29.41% frequency with 3 species, Ascomycota were least frequent and represented by only one species whereas mitosporic fungi showed 94.11% frequency and were represented by 17 species. Mycological analysis of Udhampur samples revealed an association of 19 fungal species with *Aspergillusflavus* dominated over other fungal species with a heavy load of $5.7x10^3$-$7.2\ x10^3$cfu/g. Likewise, samples procured from the markets of Rajouri and Poonch district supported the growth of 15 and 11 fungal species respectively.

The presence of a wide range of storage fungi could be ascribed to their better adaptations to the storage environment whereas, recovery of a large number of field fungi from the stored market samples reflects the poor and inadequate drying of fruits of *Diospyros lotus*. Susceptibility of dried fruits to such a diverse group of mycobiota also suggests that unhygienic and unscientific post-harvest handling operations including storage, transportation and marketing predispose them to undesirable mould infestation. It also clearly depicts that this substrate provide ample nutrients to the surface invading fungi. Various types of dried fruits have been investigated thoroughly world over for mycoflora (Zohri and Abdel-Gawad, 1993; Abdel-Sater and Saber 1999; Hosseini and Bagheri, 2012; Spencer, 2012; Khare *et al*., 2013; Tournas *et al*., 2015) but the incidence of mycoflora on dried date plum persimmons, in particularly, is only scantly reported. Although fungi associatedwith postharvest decay of persimmons (*Diospyroskaki*, *D. lotus*) were studied (Moreau, 1944-45; Palou *et al*., 2009, 2013). However, a few reportsare available on the incidence of mycoflora in dried date plum persimmons (Gunduz *et al*., 2017).

Mycotoxin analysis

The samples analyzed showed the presence of toxigenic fungi which indicate the potential for the presence of mycotoxins in dried fruits. Therefore, the dried date plum persimmons were further screened for natural contamination with aflatoxins B_1, B_2, G_1 and G_2, ochratoxin A and patulin. Of the 65 samples

analyzed, 49 samples showed widespread occurrence of different mycotoxins.The most prevalent was aflatoxins followed by ochratoxin A and patulin (table3). Among AFs, AFB_1, the most toxic one, was most frequently detected in the 61.53% aflatoxin positive samples upto the levels of 798.34µg/kg. AFB_2 was the next abundant mycotoxin (27.69%) with values ranging between 3.45 and 650.67µg/kg. G-type AFs were detected less frequently. Comparative analysis of aflatoxin contamination suggests that the highest frequency of AFB_1 was in Jammu samples with a mean concentration of 291.21±163.0µg/kg. These samples were also contaminated with high level for AFB_2, AFG_1 and AFG_2. The amount of aflatoxin B_1 and B_2 detected from Poonch samples was very low with a mean concentration of 0.78±98.78µg/kg and 6.56-25.89µg/kg respectively. This can be ascribed to the fact that aflatoxigenic fungi compete poorly under cool conditions and their aggressiveness in such areas is low in comparison to warmer regions (Cotty and Garcia, 2007). Considering the significance of OTA, its analysis in the dried fruits showed that 31 (47.69%) out of 65 samples were positive to this toxin with levels ranging from 0.76-432.09µg/kg. OTA occurred with a relatively high incidence in samples collected from Udhampur district (10 samples) followed by market samples collected from Jammu (117.20±98.67µg/kg) while the least contamination was detected in samples procured from Rajouri and Poonch districts (6.34±213.90µg/kg and 0.86-145.87µg/kg respectively).Patulin was presented in 30.76 percent samples but with a low level of concentration (0.89-123µg/kg). PAT occurred in higher incidence in Jammu samples and the level of toxin varied from 2.34-98.76µg/kg. The results of the present study indicate that samples contained aflatoxins beyond the maximum permissible limit of 4ng/g fixed by European Commission for total aflatoxins in dried fruits for human consumption (EC, 2006). Likewise, the samples were found laden with OTA and PAT exceeding the maximum tolerable limits (MTL) of 10µg/kg and 50µg/kg for OTA and PAT respectively fixed by European Commission.

This study showed that the market samples of dried fruits of *Diospyros lotus* are frequently contaminated with several toxicologically significant mycotoxins. The high incidence of different mycotoxins in the present finding depends on various factors such as the commodity, climatic conditions, agricultural practices, storage conditions and seasonal variances (Warth *et al*., 2012). Since these dried fruits are usually consumed without processing, unlike other food commodities which may undergo some level of processing before consumption that may enhance the vulnerability of consumers to these toxic contaminants. It is also pertinent to mention here that these dried fruits are commonly stored in jute bags and sold in small groceries exposed to air, moisture and dust. The legislated mycotoxins in dried fruits (AFs, OTA) also exceeded the established maximum permissible limits, which is a matter of concern and, thus, acquires much significance.Previously, various researchers have also reported mycotoxin contamination in various dried fruits and nuts including cashewnuts, hazelnuts, Brazilnuts, figs, dates, dried plum, dried apricots and almonds in amounts exceeding permissible limits set by European Union Commission (Celik and Ozturk, 2000; Hosseini *et al*., 2012; Karaca and Nas, 2006; Azaiez *et al*., 2015; Essawet *et al*., 2017; Gupta *et al*., 2017).The presence of aflatoxins, ochratoxin A and patulin in these samples directs that they are not entirely safe for human consumption owing to their properties to induce severe acute and chronic toxicity even at low dose levels. Subsequently, periodic monitoring and the imposition of appropriate quality control measures should be encouraged to reduce the invasion and colonization by storage and toxigenic moulds and in turn reduce the risk of mycotoxicoses.

Table 1. Quantitative estimation of mycotoxins

Mycotxins Estimated	**Mobile Phase used for HPLC analysis**	**Detector UV/VIS (Wavelength)**	**Elution Time (Minutes)**	**Modified Methods**
Aflatoxins(AFT)	Water: Acetonitrile: Methanol (54:34:12v/v/v)	365nm	B1: 6.484; B2: 5.740; G1: 5.453; G2: 4.884	Rohman and Triwahyudi, 2008
Ochratoxin A(OTA)	Acetonitrile:Water (50:50v/v)	220nm	8.431	Hackbart*etal*., 2012
Patulin (PAT)	Acetonitrile:Water (8:92v/v)	276nm	7.969	Beretta *etal*. 2000

Table 2. Frequency percent and total colony count of fungal species recovered from dried date plum persimmons

S.No	**Name of the fungal species**	**JammuCfu/g×10^3**			**RajouriCfu/g×10^3**			**PoonchCfu/g×10^3**			**UdhampurCfu/g×10^3**		
		% Freq.	**CDA**	**DRBC**	**% Freq.**	**CDA**	**DRBC**	**% Freq.**	**CDA**	**DRBC**	**% Freq.**	**CDA**	**DRBC**
Zygomycota													
1	*Mucormucedo*	23.52	0.5	1.6	-	-	-	12.5	-	1.5	25.0	2.2	1.2

2	*Rhizopusstolonifer*	5.8	-	0.8	12.5	-	0.5	6.25	0.9	-	18.75	2.3	-
3	*Syncephalastrumracemosum*	11.76	-	6.4	18.75	-	-	-	-	-	-	-	-
	Ascomycota												
4	*Eurotiumchevalieri*	41.17	2.3	3.3	12.5	-	1.3	-	-	-	-	-	-
5	*E. rubrum*	-	-	-	-	-	-	-	-	-	12.5	1.2	-
	Mitosporic fungi												
6	*Alternariaalternata*	29.41	2.3	1.2	37.50	0.9	1.9	-	-	-	21.53	4.5	2.3
7	*Aspergillusaculeatus*	-	-	-	12.5	-	1.3	-	-	-	-	-	-
8	*A. flavus*	88.23	8.3	6.7	81.25	1.2	4.5	68.75	2.5	4.2	87.5	5.7	7.2
9	*A. fumigates*	23.52	2.7	3.4	18.75	1.2	1.3	-	-	-	12.5	-	1.3
10	*A. japonicas*	5.8	7.9	-	25.0	0.3	0.2	-	-	-	37.5	1.9	0.9
11	*A. nidulans*	17.6	2.4	-	-	-	-	7.69	-	1.2	-	-	-
12	*A. niger*	47.05	3.9	3.6	56.25	1.5	6.4	21.53	1.3	0.9	25.0	1.8	3.4
13	*A. ochraceus*	17.8	0.8	4.5	43.75	-	2.5	18.75	1.5	2.3	31.25	-	3.9
14	*A. oryzae*	11.76	-	9.8	-	-	-	-			-	-	-
15	*A. parasiticus*	41.23	-	7.9	21.53	2.3	-	-			43.75	0.9	2.1
16	*A. sydowii*	5.8	-	2.56	-	-	-	-	-	-	-	-	-
17	*A. tamari*	-	-	-	25.0	-	1.7	6.25	2.1	1.2	-	-	-
18	*A. terreus*	-	-	-	-	-	-	-	-	-	25.0	-	0.6
19	*A. versicolor*	-	-	-	-	-	-	-	-	-	12.5	-	2.4
20	*A. wentii*	11.76	-	3.5	-	-	-	-	-	-	-	-	-
21	*Curvularialunata*	52.94	0.6	2.5	-	3.2	1.5	37.5	1.9	3.2	56.25	-	3.7
22	*Penicilliumaurantiogriseum*	-	1.2	3.5	12.5	-	2.1	-	-	-	6.25	2.1	-
23	*P. chrysogenum*	23.52	1.9	2.5	37.5	-	2.4	-	-	-	12.5	2.3	0.5
24	*P. citrinum*	-	-	-	-	-	-	18.75	2.3	5.6	6.5	-	1.3
25	*P. corylophilum*	5.8	-	4.3	-	-	-	-	-	-	-	-	-
26	*P. expansum*	41.17	1.5	4.1		3.4	-	25.0	-	1.3	18.75	-	2.1
27	*P. fellutanum*	-	-	-	-	-	-	-	-	-	12.5	0.4	0.5
28	*P. purpurogenum*	35.29	2.3	-	-	-	-	-	-	-	18.75	1.7	0.9
29	*P. verrucosum*	-	-	-	-	-	-	-	-	-	-	-	-
30	*Scopulariopsisbrevicaulis*	5.8	-	1.8	-	-	-	-	-	-	-	-	-
31	*S. brumptii*	-	-	-	-	-	-	6.25	1.2	0.9	-	-	-
Number of fungal species recovered		21	14	19	15	8	13	11	8	10	19	12	16

- Not detected

Table 3. Mycotoxin contamination in dried date plum persimmons

Mycotoxins	**Jammu (n=17)**		**Rajouri (n=16)**		**Poonch (n=16)**		**Udhampur (n=16)**	
	Number of positive samples	**Mean level of contamination**	**Number of positive samples**	**Mean level of contamination**	**Number of positive samples**	**Mean level of contamination**	**Number of positive samples**	**Mean level of contamination**
Aflatoxin B_1	12	1.50-798.34 (291.21±163.0)	9	2.54-214.54 (90.05±45.34)	8	0.78-98.78 (76.98±556.8)	11	1.23-540.45 (234.5±122.45)
Aflatoxin B_2	6	3.65-650.67 (152.55±115.77)	4	12.34-167.45 (64.76±234.4)	3	6.56-25.89 (15.78±45.89)	5	3.45-234.78 (114.67±34.56)
Aflatoxin G_1	4	11.76-196.56 (57.67±123.4)	6	8.76-156.78 (51.89±67.78)	-	-	3	2.87-45.78 (34.67±34.98)
Aflatoxin G_2	5	2.32-153.37 (87.34±12.54)	-	-	-	-	4	0.98-56.456 (45.78±23.67)
Ochratoxin A	8	0.76-345.76 (117±98.67)	6	6.34-213.90 (93.90±78.9)	7	0.86-145.87 (87.56±23.09)	10	12.45-432.09 (189±45.78)
Patulin	7	2.34-123.90 (70.81±78.79)	4	0.89-84.56 (67.98±233.90)	4	13.45-99.76 (56.67±23.9)	5	0.98-98.76 (78.67±456.77)

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