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CHARACTERIZATION AND EVALUATION OF FUNGAL FLORA ASSOCIATED WITH AFLATOXINS IN DRY PLUM BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

Plum from Rosaceae family is a temperate fruit with world production 11,758,135 metric tons and around 49,800 tons production in Pakistan. The present study aims to evaluate the incidence of myco-flora associated with dried plum causing aflatoxins that are hazardous to human health. Plum fruit was randomly collected from commercial fruit markets of Rawalpindi district Punjab, Pakistan, district Islamabad Pakistan and Gilgit Baltistan Pakistan. Fungi was isolated by using dilution plating method and mycotoxins were characterized by modified multi-mycotoxin technique. High Performance Liquid Chromatography was conducted for evaluation of quantitative and qualitative analysis of mycotoxins (aflatoxins). TFA and FLD detectionwas carried out by 360 nm and 440 nm excitation and emission wavelength. Four fungal genera were identified viz; *Aspergillus flavus, Mucorfragilis, Penicillium sp*.,and*Fusarium sp*., Furthermore, 6 of the samples showed the incidence of AFB1and AFG1 contamination with the mean range of 3.95 ± 0.0142ppm.Our findings study are a matter of concern for regulatory authorities in Pakistan to implement appropriate regulatory and control measures

to alleviate the potential public health risks associated with the consumption of dry plumpsfor availability of healthy produce.

Keywords: Dried plums; Mycoflora; Aflatoxin; HPLC

INTRODUCTION

Plums have a hard pitand are considered stone fruits and belong to the genus *Prunus* in the family Rosaceae. Most commercially produced plums can be divided into one of two categories. Japanese (diploid) or European (hexaploid) kinds. Primarily *Prunus domestica*, or European plums, are adaptable to colder climates . Dried plum fruits play a crucial role in human life and are consumed widely all over the world. They are essential for maintaining health in addition to being nutritious. The majority of these are seasonal, and when there is an excess, various techniques for dehydration and preservation are used to prepare them for usage in the off-season.

The food handling industry is the single-biggest market for dried organic products. Dried natural products are habitually used as a defensive food that can emphatically affect wellbeing and personal satisfaction and give huge pharmacological advantages in breakfast grains, bread, pastries, and confectionary things. Dessert options include fresh and dried plums, as well as their seed kernels, while many additional high-value items including plum jams, jellies, squash, and juices are also available are produced and distributed on a large basis.

The world production of Plum in 2017 was 11,758,135 metric tons and production in Pakistan around 49,800 tons of production in 2017-2018The provinces of Balochistan and Khyber Pakhtunkhwa are where it is primarily farmed. Kalat, Mardan, Nowshera, Peshawar, Pishin, Quetta, and Swat are the primary plum-producing regions.

A fruit that is high in vitamins and minerals is the plum (*Prunus domestica* L.). It is greatly valued economically and widely grown for profit (Farid et al., 2008). Based on local climatic conditions and factors like water quality, chill units, and market value, plum orchards are grown throughout Khyber Pakhtunkhwa. The Khyber Pakhtunkhaw region's plum orchard nutritional surveys, however, revealed a severe micronutrient deficiency (Tariq et al., 2008).Themost well-known and significant stone fruit economically in Pakistan is the plum (*Prunus domestica*).

Each year, Pakistan produces 50,465 metric tonnes of plums (Moosa et al., 2019).Fungi, which are a natural part of the ground, contaminate a variety of foods, including dried fruits and plums. In addition to causing these nutrients to decay, this fungus infection is also to blame for mycoses and mycotoxicoses in consumers, especially those with weakened immune systems (Abbas et al., 2019).Aflatoxin are a class of mycotoxins that are produced by several filamentous fungi, including *Aspergillusflavus* and *Aspergillus parasiticus*, and are found in nature. The primary goal of this study was to check for the presence of Aflatoxin in ready-to-eat plums that were readily available locally, analyses

them for their nutritional value, and compare the effectiveness of traditional (thin-layer chromatography [TLC]) and kit-based (enzyme-linked immunosorbent assay [ELISA]) methods for aflatoxin detection in the sample (Anshida et al., 2022).

When harvesting plums, branches are shaken to letting the fruits, which are then fanned out on wheat straw, laid on rooftops, and went every so often to dry. They change to cotton clothing as they arrive at the last stage. The jute filaments or goat hairs adhere to the completed item and lower its worth when these are incidentally moved to gunny sacks (a sweeping produced using goat hair).The product loses color during the process and comes on a brownish color (Personal observation). Traditional plum harvesting and post-harvesting practices typically involve little to no hygiene precautions, which increases the risk of contamination with different filamentous fungi and subsequent Aflatoxin development.Food product contamination with Aflatoxin now poses a severe risk. ELISA appeared as an appropriate solution for rapid and sensitive detection regardless of the fact that certain methods for the identification and quantitation of toxins have been evolved due to their low concentration of poisoning in food commodities. Aflatoxin needs an analytic approach for identification and quantitation that is precise, sensitive, and simple to use. (Anshida et al., 2022).Aflatoxin are toxic secondary metabolites, and it is unavoidable for them to enter the food chain. As a result, it is crucial to find and measure Aflatoxin (Jiménez Medina et al., 2021).

Production of Aflatoxin is polymorphic in A. *flavus*, and the species as a whole exhibit relatively high genetic diversity (Drott et al., 2020).The presence of the 26 genes that make up the Aflatoxin biosynthesis gene cluster, which is responsible for the production and transportation of Aflatoxin, is correlated with the strain-specific ability of A. *flavus* to create Aflatoxin (**Pyne et al., 2012).**Aflatoxin contamination of foods and feeds is currently becoming more widely known, and earlier investigations have found A. *flavus* in Pakistani sesame. However, no comprehensive research has been done on the genetic integrity of the Aflatoxin gene cluster and the aflatoxigenic capacity of the native A. flavus population isolated from sesame seeds in Pakistan. Here we examined sesame seed the aflatoxigenic and non-aflatoxigenic capabilities of 260 isolates and performed comparative genomic analysis of 12 selected native A. *flavus* isolates from sesame seeds grown in two agro-ecological zones of the Punjab, Pakistan.

The majority of food toxicologists today are focused on ways to reduce mycotoxin exposure and have them removed from human diets. Ochratoxin A (OTA), B, and C are toxic secondary metabolites generated by several mould species of the genera *Aspergillus* and *Penicillium*, with some chemical changes in structure (Iqbal et al., 2014). They are a class of derivatives of isocoumarin. The most prevalent kind of ochratoxins is OTA. Ochratoxin production is triggered by high humidity and temperature, unseasonal rains during harvest, and flash floods. Fungi that are involved in the formation of OTA include *Aspergillus ochraceus, Aspergillus alliaceus, Aspergillus sclerotiorum, Aspergillus niger, Aspergillus*

sulphureus, Aspergillus albertensis, Aspergillus auricomus, Aspergillus wentii, Aspergillus carbonarius, Aspergillus wester (Iqbal et al., 2018).

Molds and ochratoxins may infect different agricultural products in the human food chain growing season or after harvest. A wide range of agricultural products, including cereals and their derivatives (coffee, cocoa, oilseeds, and nuts), spices, fruits, dried fruits, and their juices, alcoholic beverages (wine and beer), and a wide range of animal products, such as milk and dairy goods, meat, and spices, can be contaminated with ochratoxins (Masood et al., 2015).

Managing the mold growth and aflatoxin production in stored produce is a challenging concern for food safety and public health. Food storage techniques are intended to limit the growth of spoilage microorganisms by providing mechanical barriers and unfavorable environmental conditions (Diarra andAmoah, 2019). In Pakistan, information available regarding the effects of storage conditions on the levels of aflatoxins in dry fruits is very limited, although studies have been carried out to evaluate aflatoxin contamination in dry fruits in Pakistan (Asghar *et al*., 2017) and (Ali *et al.,* 2020).

The present study aimed to determine the natural occurrence of aflatoxins in dry plumpsfrom three areas, Rawalpindi, Islamabad and Gilgit Baltistan, Pakistan by High Performance Liquid Chromatography.

MATERIALS AND METHODS

Sample Collection

Dried Plums samples were collected from different regions of Rawalpindi and Islamabad as in Table 3.1 (General store and open markets. (Committee chowk, Jinnah super market, Kheyabanjohar, Sabzimandi, Saddar Rawalpindi, Raja bazaar Rawalpindi, Sunday bazaar Islamabad, Sadiqabad Rawalpindi, Nli market Gilgit, Gilgitbaseen, Sadiqabad Transformer chowk, I-10 Markaz Islamabad. Total Twelve dried plum samples were collected in sterilized polyethylene bags randomly, labeled a stored at low temperature at 4°C for further mycobial assay in Fungal Plant Pathology laboratory in Department of Plant Pathology at Arid Agriculture university Rawalpindi.

Mycological Analysis

The moulds in the dried plums were isolated by dilution plating method. A further processed for identification.

Dilution Plating Method

The Dilution Plating Method (Gnonlonfin et al. (2008) was use to isolate the mycoflora affiliated with several plum samples. About 10 g plums samples were taken in pestle mortar grinded thoroughly before making paste, plum paste was added in 250 ml Erlenmeyer flask containing sterilized distilled water and stirred continuously using rotary shaker at 200 rotations per minute for 30 min. With distilled water that has beensterilized, residue was diluted. Following that, aliquots (1 ml) of each dilution were put into petri dishes with Potato Dextrose Agar (PDA). The proportion abundance of recovered fungus species was determined after five days of incubation at 28.2 °C using five duplicate plates per medium.

Identification of fungal cultures

The collected fungal species were grown on PDA media and identified based on their cultural and micromorphological traits with the aid of pertinent literature and suggested keys.

Mycotoxins are extracted through naturally contaminated dried plums. To identify and estimate mycotoxins in naturally contaminated market specimens of dried plums, a modified multi-mycotoxin approach developed by Roberts and Patterson will be used. In a pestle and mortar, the dry materials were thoroughly mashed. In a 250 mili Liter Erlenmeyer flask filled with a 250 mili Liter combination of distilled water, 10 grams of the crushed sample was added.PDA media was used for growth of *Aspergillus flauvs,* three replications were taken of the sample R1, R2 and R3 respectively. Then transferred these samples to incubator for 5 days at 28C temperature. Growth of fungus can be identified by two methods by using cultures and microscopic technique.

Qualitative and quantitative estimation of Mycotoxins and Aflatoxin

Different amounts of the aflatoxins stock standard were placed in 2 mL volume Eppendorf tubes and were let to dry under a gentle stream of N_2. After drying, samples were derivative using trifluoroacetic acid (TFA) as described by the AOAC. The HPLC system used for AF analysis was an Agilent 1200 series system (Agilent, Berks., UK) with a fluorescence detector (FLD, G1321A, Agilent), an auto sampler (ALS, G1329, Agilent). Analysis was performed in the isocratic mode and the mobile phase was ethanol **(30v)** using a flow rate of 1 mL min−1. FLD detection was performed using 360 nm and 440 nm excitation and emission wavelengths respectively; only one column C-18 was used.

Total 12 Samples of dried plum from were used for further process. All plum samples, weighing approx. 10 g, were removed from these samples using a cork borer. They were placed in previously weighed 2 mL volume Eppendorf tubes. A total of 3 replicates per treatment were collected, weighed, and immediately frozen at −20 °C and stored.

For Aflatoxin extraction 800 µl chloroform was added to each Eppendorf and shaken well for 30 min. The chloroform extract was transferred to a new vial and dried gently under air. Afterwards samples were derivative using TFA as described by the AOAC.

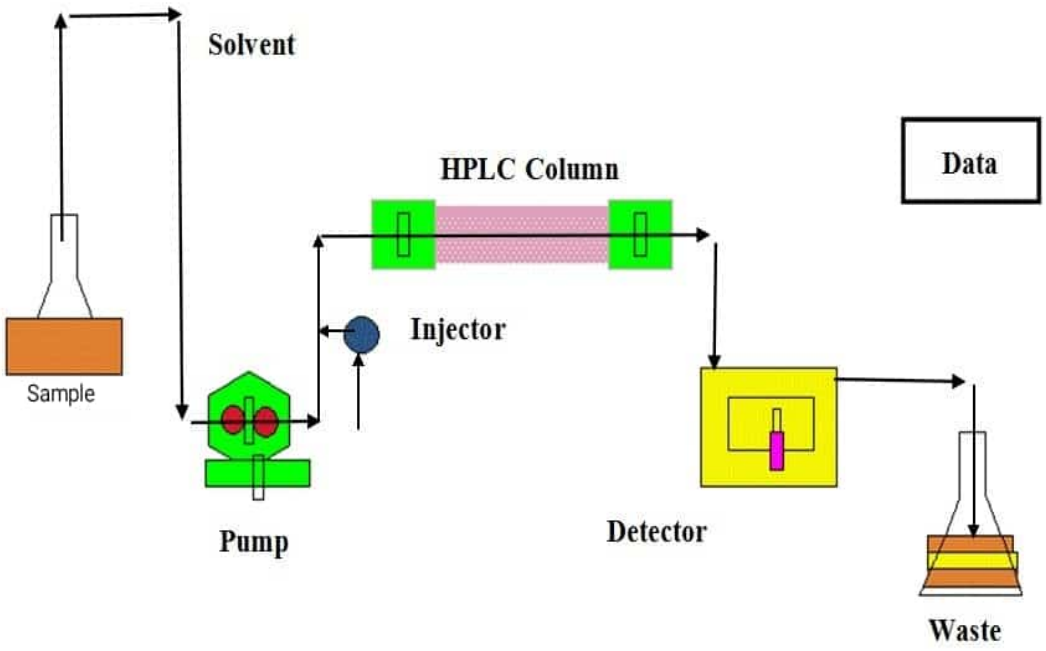

Figure 1. Pictorial view of HPLC Apparatus

High Performance Liquid Chromatography (HPLC) is a process of separating components in a liquid mixture. A liquid sample is injected into a stream of solvent (**mobile phase**) flowing through a column packed with a separation medium (**stationary phase**). Sample components separate from one another by a process of **differential migration** as they flow through the column. As bands emerge from the column, flow carries them to one or more detectors which deliver a voltage response as a function of time. This is called a **chromatogram**. For each peak, the time at which it emerges identifies the sample constituent with respect to a standard. The peak's area represents the quantity.

All of the tests were triple repeated, and statistics 8.1 software were used to statistically assess the statistical models. The fixed P - 0.05 threshold for statistical significance is used.

RESULTS AND DISCUSSION

Total Twelve dried plum samples were collected in sterilized polyethylene bags randomly, labeled a stored at low temperature at 4°C for further mycobial assay in Fungal Plant Pathology laboratory in Department of Plant Pathology at Arid Agriculture university Rawalpindi.

Table1. Collection of sample

Sample	Sample Size	Location
1	10g	Committee Chowk
2	10g	Khayaban e Johar

3	10g	SabziMandi, Isb
4	10g	Saddar, Rwp
5	10g	Raja Bazar, Rwp
6	10g	GilgitBaseen
7	10g	Jinnah Super, Isb
8	10g	Sunday Bazar, H9
9	10g	Sadiqabad, Rwp
10	10g	Sadiqabad, TC
11	10g	I10 Market, Isb
12	10g	NLI Market, Gilgit

MYCOLOGICAL ANALYSIS

Mycoflora Associated with Dried Plumps

In the current researches, dilution plating and conventional blotter methods were used to detect eight fungus isolates from the different genera *Aspergillus flavus, Mucor fragilis, Penicillium sp., Fusarium sp., mentioned in* (Table 4.2). These species contributed as important elements of dried plums, constituting a taxonomic group of worldwide fungal organisms that can utilize

practically any organic substrate granted an appropriate oxygen, temperature, and relative humidity storage environment. It builds up harmful secondary metabolites. On black sultanas, white sultanas, plums, and dried plums, a recent research from Brazil also found the presence of mycoflora, including *AspergillusNiger*, A. *carbonarius*, A. *ochraceus*, A. *flavus*., and *Fusarium* sp. These findings are consistent with a study that found that just 1-3 of the less than 10 fungus species prevalent in food nuts 313 dominate and cause 314 deleterious effects (Filtenborg et al., 2004). From 41.7%, 16.7%, and 16.7% of the walnut 316 samples, the species *Mucor*, were isolated, respectively.

MORPHOLOGICALY IDENTIFICATION OF *ASPERGILLUSFLAVUS*

Total 12 samples were taken for analysis of Aflatoxin in Plum in which frequency distribution of fungal flora was 50%. Percentage of fungus sp. were *Aspergillus flavus, Aspergillus niger, A. carbonarius, A. ochraceus,* and *Fusarium* sp. were 80 %, 40 %, 20 %, 10 %, 5 % and 2 % respectively as shown in Table 2.

Table 2. Frequency distribution of fungal Flora

No. Of Samples	Frequency Distribution of fungal Flora		Percentage of Fungal Sp.
12	50 percent	*Aspergillus. flavus*	80%
		Aspergillus. niger	40%
		A. carbonarius	20%
		A. ochraceus	10%
		Fusarium sp.	2%

Over 180 identified anamorphic species and nine distinct genera with verified teleomorphs make up the sizable genus Aspergillus species. The seven subgenera that comprise the genus are further classified into Sections. As with

other fungi, Aspergillus has a complex and evolving taxonomy. The genus is easily recognised by its distinctive conidiophore, but scientists still struggle to separate and identify different species because they have historically relied on a range of morphological traits. The colour of the conidia and mycelium, colony diameter, colony reverse colour, production of exudates and soluble pigments, presence of sclerotia and cleistothecia, and other macromorphological traits are taken into consideration. Characterization of the micro morphology is primarily based on the serration, vesicle size and form, conidia and stripe morphology, presence of Huller cells, and features have to be determined under standardised laboratory conditions by trained scientists in order to obtain an accurate identification. There are numerous Aspergillus taxonomy keys and guides available.

It is well knowledge that a number of Aspergillus species are regarded as the most prominent toxigenic varieties. Of particular concern was Aspergillus flavus, a well-known generator of aflatoxin, which infected 50% of them. The majority of the Aspergillus species found during this inquiry were previously isolated from various dried plums. Previously, when researching the mycoflora of dried samples of plum and plum raisin in Egypt, Zohri and Abdel-Gawad isolated 55 species and two variations from 23 genera.

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In all 12 samples of dried plums from different locations of three cities Rawalpindi, Islamabad and Gilgit, only 6 samples were found toxigenic . The results depicted that by comparing these sites, the samples collected from Rawalpindi showed high incidence as compared to other sites (Islamabad and Gilgit). The dried plum samples from Gilgit showed minimum level of Aflatoxins. This might be due to the storage conditions of these plumps which may leads to cause the occurrence of Aflatoxin. (*Aspergillus flavus)*. Remaining six samples foundfree of Aflatoxin (Fig.2).

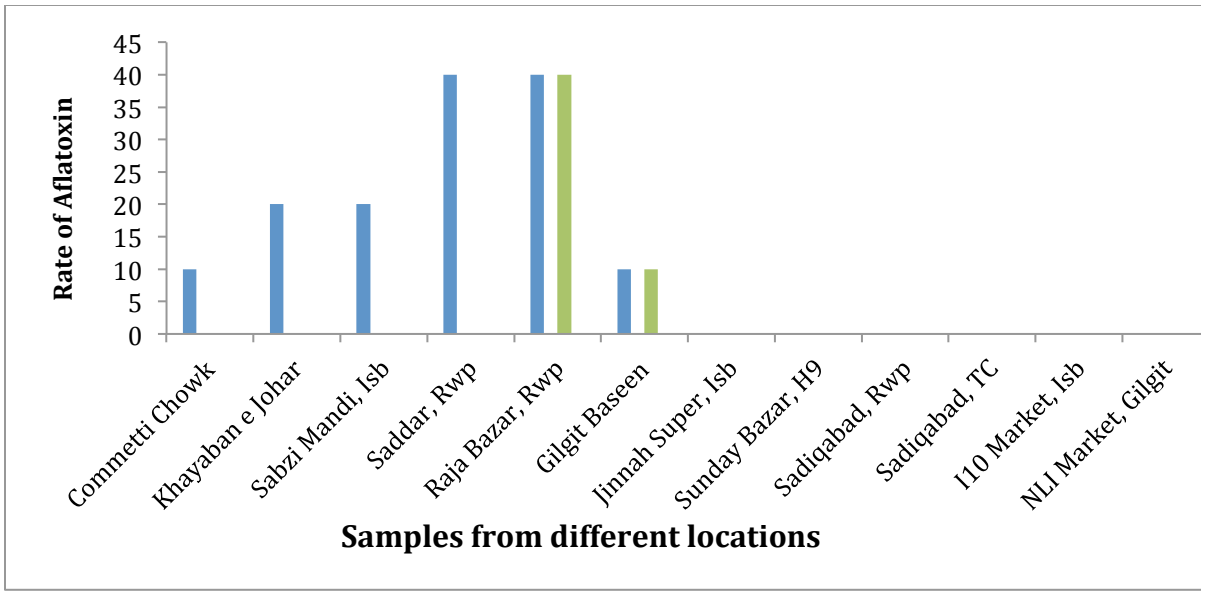

Figure 2. Occurrence of Aflatoxins on different dried plum samples collected from different areas of Rawalpindi.

High Performance Liquid Chromatography (HPLC) results showed no occurrence of any Aflatoxins on dried plum viz, Sunday bazaar H-9 Islamabad,Jinnah Super, Sadiqabad Rawalpindi, NLI Market Gilgit, Sadiqabad Transformer Chowk, I-10 Market Islamabad. While as shown in table(4.3) the committee chowk sample found B1 Aflatoxin at the rate of 10 ppm while Aflatoxin B2, G1 and G2 had not been reported on processed samples analysis under HPLC. KheyabanyeJohar, I-8 Islamabad showed B1 Aflatoxin at the rate of 20 ppm while Aflatoxin B2, G1 and G2 had not reported any occurrence on samples same results had been found with Sabzimandi sample. The rate of Aflatoxins on Sadar Rawalpindi samples had been determined B1 at the rate of 40 ppm while Alfatoxin B2, G1 and G2 had not showed any occurrence on samples.

Results shown in table (4.3) at the Raja Bazar B1 and G1 Aflatoxin at the rate of 40 ppm while Alfatoxin B2, and G2 had not showed any occurrence on sample. Results shown in table at the GLT Baseen B1 and G1 Aflatoxin at the rate of 10 ppm while Alfatoxin B2, and G2 had not showed any occurrence on samples.

Reason may be all plum stock in that shops and markets were well kept at optimum temperature and at less humidity. Because both factors play an important role in development of pathogen or in growth of pathogen. Stock were stored in boxes so there is less chances of contamination by other products. They all have new stock of season which is harvested recently.

Table 3. Presence of Aflatoxin in all samples collected from different areas of Rawalpindi, Islamabad and Gilgit.

Sr. No	Samples	Aflatoxin B1	Aflatoxin B2	Aflatoxin G1	Aflatoxin G2
1	Committee Chowk	10ppm	Not-detected	Not-detected	Not-detected
2	Khayaban e Johar	20ppm	Not-detected	Not-detected	Not-detected
3	SabziMandi, Isb	20ppm	Not-detected	Not-detected	Not-detected
4	Saddar, Rwp	40ppm	Not-detected	Not-detected	Not-detected
5	Raja Bazar, Rwp	40ppm	Not-detected	40ppm	Not-detected
6	GilgitBaseen	10ppm	Not-detected	10ppm	Not-detected

7	Jinnah Super, Isb	Not-detected	Not-detected	Not-detected	Not-detected
8	Sunday Bazar, H9	Not-detected	Not-detected	Not-detected	Not-detected
9	Sadiqabad, Rwp	Not-detected	Not-detected	Not-detected	Not-detected
10	Sadiqabad, TC	Not-detected	Not-detected	Not-detected	Not-detected
11	I10 Market, Isb	Not-detected	Not-detected	Not-detected	Not-detected
12	NLI Market, Gilgit	Not-detected	Not-detected	Not-detected	Not-detected

Results of Data Analysis:

Table 4. Analysis of variance ANOVA for Result

Source	DF	SS	MS	F	P

Aflatoxin	3	1089.58	363.194	3.94	0.0142
Error	44	4058.33	92.235		
Total	47	5147.92			

Grand Mean 3.9583

CV 242.62

Component of variance for between groups 22.5800

Effective cell size 12.0

Table 5. LSD for above ANOVA table

Aflatoxin	Mean
B1	11.667
B2	0.0000
G1	4.1667
G2	0.0000
Observations per Mean	12

Standard Error of a Mean

2.7724

Std Error (Diff of 2 Means)

3.9208

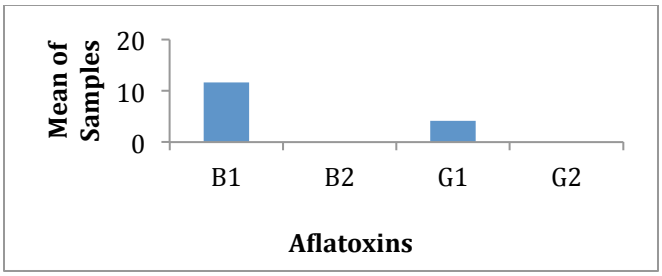

Figure 3. Std. Error

In table 3 shows that collected samples from 12 different places and analysis was done by HPLC test and we have aflatoxins detected in six samples and apply statistics on the value. The (DF) degree of freedom is 3, some of square (SS) is 1089.58, Mean square (MS) is 363.194, Frequency (F) is 3.94 and level of significance is 0.0142 and then lastly, we find the grand means which is 3.958.

The after effects of the current review demonstrate that dried plum tests contained aflatoxins past the most extreme admissible constraint of (either mean worth of Aflatoxin or Sum Value fixed all out aflatoxins in dried natural products for human utilization utilizing HPLC detected AFB1, AFB2, AFG1 and AFG2 with mean value of 11.667, 0, 4.667 and 0 ppm respectively in dried Plum.

Table 6. LSD All Pairwise Comparisons Test of Result by Replications

Aflatoxin	Mean	Homogeneous Groups
B1	11.667	A
G1	4.1667	AB
B2	0.0000	B
G2	0.0000	B

Alpha 0.05 Standard Error for Comparison 3.9208

Critical T Value 2.015 Critical Value for Comparison 7.9018

The event of Aflatoxin B1, B2, G1 and G2 was explored by HPLC in dried plum. Tests of the all-out 12 examples evaluated for the presence of mycotoxins, 6 examples were viewed as sure for AFB1 and AFG1 pollution with the mean scope of 3.95 ± 0.0142ppm. Be that as it may, 6 of the examples showed the rate of AFB1and AFG1 (Table 4.4). The aftereffects of the current review show that dried plum tests contained aflatoxin past the most extreme admissible constraint of 50 mg/g fixed aflatoxins in dried natural products for human utilization.

DISCUSSION

The world production of Plum in 2017 was 11,758,135 metric tons and its production in Pakistan around 49,800 tons of production in 2017-2018. It is mostly grown in the province of Balochistan and Khyber Pakhtunkhwa. The main producing areas of plum are Kalat, Mardan, Nowshera, Peshawar, Pishin, Quetta and Swat. To evaluate the mycoflora associated with dried plump causing aflatoxins we designed present study. Aflatoxins are derivatives synthesized through the polyketide pathway by several fungal species, mainly Aspergillus flavus, A. parasiticus, and A. nomius, widely distributed contaminants of a variety of agri-food commodities (Smith *et al.,* 2019). In this study, twenty-five samples (samples size) of dried plums from different markets were evaluated by applying the International Seed Testing association method (1985). Aflatoxins assay was employed by Using the HPLC the occurrence of aflatoxin B1, B2, G1 and G2 was investigated by HPLC in dried plum. samples of the total 12 samples screened for the presence of mycotoxins, 6 samples were found to be positive for AFB1 and

AFG1 contamination with the mean range of 3.95 ± 0.0142ppm. However, 6 of the samples showed the incidence of AFB1and AFG1 (Tables 4-3). Dried fruits and nuts are particularly susceptible to fungal contamination and aflatoxin accumulation during the pre- and post-harvest production processes (Wu *et al.,* 2018). The results of the present study indicate that dried plum samples contained aflatoxin beyond the maximum permissible limit of 4 mg/g fixed by European Commission for total aflatoxins in dried fruits for human consumption. High concentration of these mycotoxins poses a serious threat as natural contaminant of dried fruits. In their studies on aflatoxin contamination, Morton et al. demonstrated that dried plums possessed highest potential for aflatoxin along with dried figs. In another study from Egypt, dried fruits (plum, plum and raisin) wereassayed for the natural occurrence of aflatoxin B1, B2, G1, G2, ochratoxin and patulin and the concentration of OTA in dried plums ranged between 50 and 110 lg/kg. However, no other mycotoxins were detected in these dried fruits. Similarly, (Celik and Ozturk, 2000) studied and revealed that plums dried on soil and tarp and later treated with sulphur dioxide were contaminated with aflatoxin B1 and G1 in the range of 0.10–1.47 lg/kg while in untreated dried samples the range of aflatoxin was 0.35–1.27 lg/kg respectively. Likewise, available reports from Turkey suggest that AFB1 was present in 3 out of 15 dried plum samples with mean value of 1.44 lg/kg (Janati et al., 2010) from Iran investigated the presence of two toxins i.e. Aflatoxin and ochratoxin A using immunoaffinity column clean up and HPLC and detected AFB1, AFB2, AFG1, OTA with mean value of 0.88, 0.32, 0.20, 2.83 ppm respectively in dried plums.Various studies

have reported on the prevalence of aflatoxins in dry fruits from different parts of the world (Zahra *et al.,* 2019) and (Wang *et al.,* 2018) and (Kang *et al.,* 2010). In all 12 samples of dried plums from different locations of three cities Rawalpindi, Islamabad and from Gilgit, only 6 samples were infected by Aflatoxin B1 and G1 followed by infection observed in other 6 samples of Aflatoxin using High Performance Liquid Chromatography, AFB1, AFB2, AFG1 and AFG2 with mean values 11.667, 0, 4.667, respectively.

CONCLUSION

The level of contamination of dry plump with aflatoxins in Punjab and Gilgit Baltistan, Pakistan, was investigated in this study. Our finding revealed that dry plump from Rawalpindi and Gilgit Baltistan collected during study was contaminated with B1 and G1 followed by infection observed in other 6 samples of Aflatoxin detected during High Performance Liquid Chromatography, AFB1, AFB2, AFG1 and AFG2 with mean values 11.667, 0, 4.667, respectively. It is therefore recommended that the consumption of dry plumps must be paralleled by efficient measures for prevention and detoxification of Aflatoxin contamination for the provision of healthy and nutritious dry fruits.

CONFLICT OF INTEREST

The author declares no conflict of interest.

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Manuscript Number:	**Vol.2,Iss3.September 2022**
Manuscript Title:	CHARACTERIZATION AND EVALUATION OF FUNGAL FLORA ASSOCIAT AFLATOXINS IN DRY PLUM BY HIGH PERFORMANCE LIQUID CHROMAT

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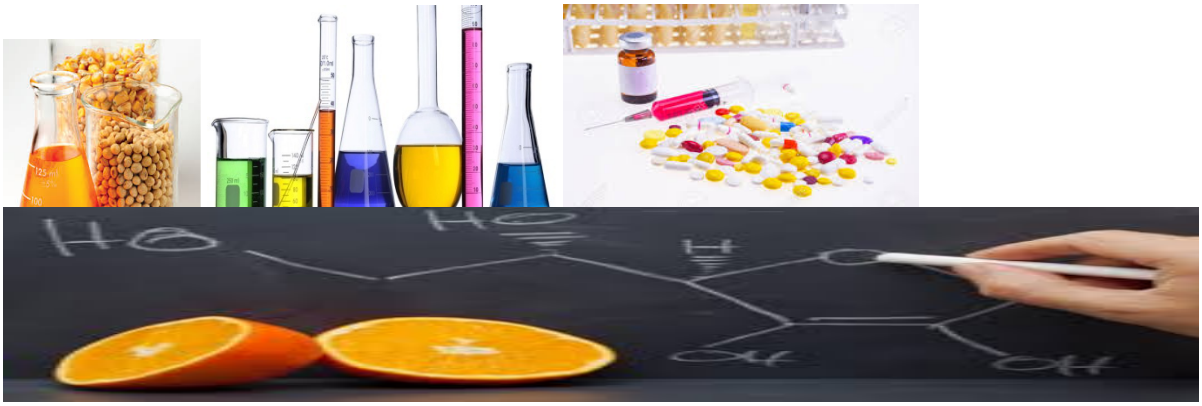

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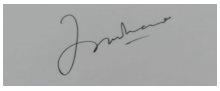

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Characterization and Evaluation of Fungal Flora Associated with Aflatoxins in Dry Plum by High Performance Liquid Chromatography

ABSTRACT

Plum from Rosaceae family is a temperate fruit with world production 11,758,135 metric tons and around 49,800 tons production in Pakistan. The present study aims to evaluate the incidence of myco-flora associated with dried plum causing aflatoxins that are hazardous to human health. Plum fruit was randomly collected from commercial fruit markets of Rawalpindi district Punjab, Pakistan, district Islamabad Pakistan and Gilgit Baltistan Pakistan. Fungi was isolated by using dilution plating method and mycotoxins were characterized by modified multi-mycotoxin technique. High Performance Liquid Chromatography was conducted for evaluation of quantitative and qualitative analysis of mycotoxins (aflatoxins). TFA and FLD detectionwas carried out by 360 nm and 440 nm excitation and emission wavelength. There were four types of fungi discovered: *Aspergillus flavus, Mucorfragilis, Penicillium sp., and Fusarium sp*. The incidence of AFB1and AFG1 contamination was also observed, with a mean range of 3.950.0142ppm across 6 samples. There are possible public health dangers linked with the consumption of dry plumps, and our research raises this issue. As a result, regulatory authorities in Pakistan should take necessary regulation and control steps to address this issue.

Keywords: Dried plums; Mycoflora; Aflatoxin; HPLC

INTRODUCTION

Plums have a hard pitand are considered stone fruits and belong to the genus *Prunus* in the family Rosaceae. Most commercially produced plums can be divided into one of two categories. Japanese (diploid) or European (hexaploid) kinds. Primarily *Prunus domestica*, or European plums, are adaptable to colder climates . Dried plum fruits play a crucial role in human life and are

consumed widely all over the world. They are essential for maintaining health in addition to being nutritious. The majority of these are seasonal, and when there is an excess, various techniques for dehydration and preservation are used to prepare them for usage in the off-season.

The food handling industry is the single-biggest market for dried organic products. Dried natural products are habitually used as a defensive food that can emphatically affect wellbeing and personal satisfaction and give huge pharmacological advantages in breakfast grains, bread, pastries, and confectionary things. Dessert options include fresh and dried plums, as well as their seed kernels, while many additional high-value items including plum jams, jellies, squash, and juices are also available are produced and distributed on a large basis. The world production of Plum in 2017 was 11,758,135 metric tons and production in Pakistan around 49,800 tons of production in 2017-2018The provinces of Balochistan and Khyber Pakhtunkhwa are where it is primarily farmed. Kalat, Mardan, Nowshera, Peshawar, Pishin, Quetta, and Swat are the primary plum-producing regions.

A fruit that is high in vitamins and minerals is the plum (*Prunus domestica* L.). It is greatly valued economically and widely grown for profit (Farid et al., 2008). Based on local climatic conditions and factors like water quality, chill units, and market value, plum orchards are grown throughout Khyber Pakhtunkhwa. The Khyber Pakhtunkhaw region's plum orchard nutritional surveys, however, revealed a severe micronutrient deficiency (Tariq et al., 2008).Themost well-known and significant stone fruit economically in Pakistan is the plum (*Prunus domestica*).

Each year, Pakistan produces 50,465 metric tonnes of plums (Moosa et al., 2019).Fungi, which are a natural part of the ground, contaminate a variety of foods, including dried fruits and plums. In addition to causing these nutrients to decay, this fungus infection is also to blame for

mycoses and mycotoxicoses in consumers, especially those with weakened immune systems (Abbas et al., 2019).Aflatoxin are a class of mycotoxins that are produced by several filamentous fungi, including *Aspergillusflavus* and *Aspergillus parasiticus*, and are found in nature. The primary goal of this study was to check for the presence of Aflatoxin in ready-to-eat plums that were readily available locally, analyses them for their nutritional value, and compare the effectiveness of traditional (thin-layer chromatography [TLC]) and kit-based (enzyme-linked immunosorbent assay [ELISA]) methods for aflatoxin detection in the sample (Anshida et al., 2022).

When harvesting plums, branches are shaken to letting the fruits, which are then fanned out on wheat straw, laid on rooftops, and went every so often to dry. They change to cotton clothing as they arrive at the last stage. The jute filaments or goat hairs adhere to the completed item and lower its worth when these are incidentally moved to gunny sacks (a sweeping produced using goat hair).The product loses color during the process and comes on a brownish color (Personal observation). Traditional plum harvesting and post-harvesting practices typically involve little to no hygiene precautions, which increases the risk of contamination with different filamentous fungi and subsequent Aflatoxin development.Food product contamination with Aflatoxin now poses a severe risk. ELISA appeared as an appropriate solution for rapid and sensitive detection regardless of the fact that certain methods for the identification and quantitation of toxins have been evolved due to their low concentration of poisoning in food commodities. Aflatoxin needs an analytic approach for identification and quantitation that is precise, sensitive, and simple to use. (Anshida et al., 2022).Aflatoxin are toxic secondary metabolites, and it is unavoidable for them to enter the food chain. As a result, it is crucial to find and measure Aflatoxin (Jiménez Medina et al., 2021).

Production of Aflatoxin is polymorphic in A. *flavus*, and the species as a whole exhibit relatively high genetic diversity (Drott et al., 2020).The presence of the 26 genes that make up the Aflatoxin biosynthesis gene cluster, which is responsible for the production and transportation of Aflatoxin, is correlated with the strain-specific ability of A. *flavus* to create Aflatoxin (**Pyne et al., 2012).**Aflatoxin contamination of foods and feeds is currently becoming more widely known, and earlier investigations have found A. *flavus* in Pakistani sesame. However, the genetic stability of the aflatoxin gene cluster and the aflatoxigenic potential of the indigenous A. flavus population isolated from sesame seeds in Pakistan have not been thoroughly studied. In this study, we compared the genomes of 12 randomly selected native A. flavus isolates from sesame seeds grown in two agro-ecological zones of the Punjab, Pakistan, to determine whether or not they were aflatoxigenic. A total of 260 isolates were tested for their aflatoxigenic and non-aflatoxigenic potentials.

The majority of food toxicologists today are focused on ways to reduce mycotoxin exposure and have them removed from human diets. Ochratoxin A (OTA), B, and C are toxic secondary metabolites generated by several mould species of the genera *Aspergillus* and *Penicillium*, with some chemical changes in structure (Iqbal et al., 2014). They are a class of derivatives of isocoumarin. The most prevalent kind of ochratoxins is OTA. Ochratoxin production is triggered by high humidity and temperature, unseasonal rains during harvest, and flash floods. Fungi that are involved in the formation of OTA include *Aspergillus ochraceus, Aspergillus alliaceus, Aspergillus sclerotiorum, Aspergillus niger, Aspergillus sulphureus, Aspergillus albertensis, Aspergillus auricomus, Aspergillus wentii, Aspergillus carbonarius, Aspergillus wester* (Iqbal et al., 2018).

Molds and ochratoxins may infect different agricultural products in the human food chain growing season or after harvest. A wide range of agricultural products, including cereals and their derivatives (coffee, cocoa, oilseeds, and nuts), spices, fruits, dried fruits, and their juices, alcoholic beverages (wine and beer), and a wide range of animal products, such as milk and dairy goods, meat, and spices, can be contaminated with ochratoxins **(**Masood et al., 2015**)**.

Storing fruits and vegetables presents a difficult challenge for managing mold growth and aflatoxin formation, two major threats to public health and food safety. Traditional methods of preserving food use barriers made of metal and chemicals to create an environment unsuitable for the development of spoiling germs (Diarra andAmoah, 2019). A few studies have been conducted to assess the amount of aflatoxin contamination in dry fruits in Pakistan, however the data is few with respect to the impact of storage conditions on the levels of aflatoxins in dry fruits (Asghar *et al*., 2017) and (Ali *et al*., 2020).

The present study aimed to determine the natural occurrence of aflatoxins in dry plumpsfrom three areas, Rawalpindi, Islamabad and Gilgit Baltistan, Pakistan by High Performance Liquid Chromatography.

MATERIALS AND METHODS

Sample Collection

Dried Plums samples were collected from different regions of Rawalpindi and Islamabad as in Table 3.1 (General store and open markets. (Committee chowk, Jinnah super market, Kheyabanjohar, Sabzimandi, Saddar Rawalpindi, Raja bazaar Rawalpindi, Sunday bazaar Islamabad, Sadiqabad Rawalpindi, Nli market Gilgit, Gilgitbaseen, Sadiqabad Transformer chowk, I-10 Markaz Islamabad. Total Twelve dried plum samples were collected in sterilized

polyethylene bags randomly, labeled a stored at low temperature at 4°C for further mycobial assay in Fungal Plant Pathology laboratory in Department of Plant Pathology at Arid Agriculture university Rawalpindi.

Mycological Analysis

The moulds in the dried plums were isolated by dilution plating method. A further processed for identification.

Dilution Plating Method

The Dilution Plating Method (Gnonlonfin et al. (2008) was use to isolate the mycoflora affiliated with several plum samples. About 10 g plums samples were taken in pestle mortar grinded thoroughly before making paste, plum paste was added in 250 ml Erlenmeyer flask containing sterilized distilled water and stirred continuously using rotary shaker at 200 rotations per minute for 30 min. With distilled water that has beensterilized, residue was diluted. Following that, aliquots (1 ml) of each dilution were put into petri dishes with Potato Dextrose Agar (PDA). The proportion abundance of recovered fungus species was determined after five days of incubation at 28.2 °C using five duplicate plates per medium.

Identification of fungal cultures

The collected fungal species were grown on PDA media and identified based on their cultural and micromorphological traits with the aid of pertinent literature and suggested keys.

Mycotoxins are extracted through naturally contaminated dried plums. To identify and estimate mycotoxins in naturally contaminated market specimens of dried plums, a modified multi-mycotoxin approach developed by Roberts and Patterson will be used. In a pestle and mortar, the dry materials were thoroughly mashed. In a 250 mili Liter Erlenmeyer flask filled with a 250 mili Liter combination of distilled water, 10 grams of the crushed sample was added.PDA media was used for growth of *Aspergillus flauvs,* three replications were taken of the sample R1, R2 and R3 respectively. Then transferred these samples to incubator for 5 days at 28C temperature. Growth of fungus can be identified by two methods by using cultures and microscopic technique.

Qualitative and quantitative estimation of Mycotoxins and Aflatoxin

Aflatoxins stock standard was divided up into various volumes of 2 mL Eppendorf tubes and dried out in a slow-moving stream of N2. Following drying, samples were processed by the AOAC-recommended derivative utilizing trifluoroacetic acid (TFA). The Agilent 1200 series system (Agilent, Berks., UK) was equipped with a fluorescence detector (FLD, G1321A, Agilent), an auto sampler, and a UV-vis-NIR spectrophotometer for AF analysis (ALS, G1329, Agilent). For this analysis, we used a flow rate of 1 mL per minute and an isocratic mode with ethanol as the mobile phase (at 30 volts). Only one column of C-18 was employed for the FLD detection process, and the excitation and emission wavelengths were 360 nm and 440 nm, respectively.

As many as 12 different dried plum samples were utilized for analysis. A cork borer was used to extract a total of 10 g of plum samples from each of these bottles. They were weighed ahead of time and then transferred to Eppendorf tubes with a 2 mL capacity. We collected, weighed, and kept three duplicates per treatment, each at 20 °C.

Each Eppendorf was filled with 800 l of chloroform and agitated vigorously for 30 minutes to facilitate Aflatoxin extraction. To dry the chloroform extract, it was re-contained in a fresh vial and placed in the air. After that, TFA was used to derive the samples, following AOAC guidelines.

High Performance Liquid Chromatography (HPLC) is a technique used to isolate certain substances from a complex solution. For this technique, a sample of liquid is injected into a moving stream of solvent (the mobile phase) that is passing through a column that is filled with a separation medium (stationary phase). As the sample moves through the column, the various parts of the sample migrate at different rates, creating separations. Whenever a band is released from the column, the flow will carry it to a detector where it will be measured in terms of voltage as a function of time. A chromatogram is what you'd name this. The appearance time of each peak may be used to determine which component in the sample is being compared to the reference. The area of the peak is a representation of the total.

All experiments were performed three times, and statistical significance was determined using the Statistical Analysis System version 8.1. We choose a standard statistical significance level of P 0.05.

RESULTS AND DISCUSSION

Total Twelve dried plum samples were collected in sterilized polyethylene bags randomly, labeled a stored at low temperature at 4°C for further mycobial assay in Fungal Plant Pathology laboratory in Department of Plant Pathology at Arid Agriculture university Rawalpindi.

MYCOLOGICAL ANALYSIS

Mycoflora Associated with Dried Plumps

In the current researches, dilution plating and conventional blotter methods were used to detect eight fungus isolates from the different genera *Aspergillus flavus, Mucor fragilis, Penicillium sp., Fusarium sp., mentioned in* (Table 4.2). These species were crucial to the development of dried plums, and they represent a taxonomic class of fungal organisms found all

over the globe that can break down almost any organic substrate given the right conditions (oxygen, temperature, and humidity) for preservation. There is a rise in potentially hazardous secondary metabolites. On black sultanas, white sultanas, plums, and dried plums, a recent research from Brazil also found the presence of mycoflora, including *AspergillusNiger*, A. *carbonarius*, A. *ochraceus*, A. *flavus*., and *Fusarium* sp. These findings are consistent with a study that found that just 1-3 of the less than 10 fungus species prevalent in food nuts 313 dominate and cause 314 deleterious effects (Filtenborg et al., 2004). From 41.7%, 16.7%, and 16.7% of the walnut 316 samples, the species *Mucor*, were isolated, respectively.

MORPHOLOGICALY IDENTIFICATION OF *ASPERGILLUSFLAVUS*

Total 12 samples were taken for analysis of Aflatoxin in Plum in which frequency distribution of fungal flora was 50%. Percentage of fungus sp. were *Aspergillus flavus, Aspergillus niger, A. carbonarius, A. ochraceus,* and *Fusarium* sp. were 80 %, 40 %, 20 %, 10 %, 5 % and 2 % respectively as shown in Table 2.

Over 180 identified anamorphic species and nine distinct genera with verified teleomorphs make up the sizable genus Aspergillus species. The seven subgenera that comprise the genus are further classified into Sections. As with other fungi, Aspergillus has a complex and evolving taxonomy. The genus is easily recognised by its distinctive conidiophore, but scientists still struggle to separate and identify different species because they have historically relied on a range of morphological traits. The colour of the conidia and mycelium, colony diameter, colony reverse colour, production of exudates and soluble pigments, presence of sclerotia and cleistothecia, and other macromorphological traits are taken into consideration. Characterization of the micro morphology is primarily based on the serration, vesicle size and form, conidia and stripe morphology, presence of Huller cells, and features have to be determined under standardised laboratory conditions by trained scientists in order to obtain an accurate identification. There are numerous Aspergillus taxonomy keys and guides available.

It is well knowledge that a number of Aspergillus species are regarded as the most prominent toxigenic varieties. Of particular concern was Aspergillus flavus, a well-known generator of aflatoxin, which infected 50% of them. The majority of the Aspergillus species found

during this inquiry were previously isolated from various dried plums. Previously, when researching the mycoflora of dried samples of plum and plum raisin in Egypt, Zohri and Abdel-Gawad isolated 55 species and two variations from 23 genera.

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

In all 12 samples of dried plums from different locations of three cities Rawalpindi, Islamabad and Gilgit, only 6 samples were found toxigenic . The results depicted that by comparing these sites, the samples collected from Rawalpindi showed high incidence as compared to other sites (Islamabad and Gilgit). The dried plum samples from Gilgit showed minimum level of Aflatoxins. This might be due to the storage conditions of these plumps which may leads to cause the occurrence of Aflatoxin. (*Aspergillus flavus)*. Remaining six samples foundfree of Aflatoxin (Fig.2).

High Performance Liquid Chromatography (HPLC) results showed no occurrence of any Aflatoxins on dried plum viz, Sunday bazaar H-9 Islamabad,Jinnah Super, Sadiqabad Rawalpindi, NLI Market Gilgit, Sadiqabad Transformer Chowk, I-10 Market Islamabad. While as shown in table(4.3) the committee chowk sample found B1 Aflatoxin at the rate of 10 ppm while Aflatoxin B2, G1 and G2 had not been reported on processed samples analysis under HPLC. KheyabanyeJohar, I-8 Islamabad showed B1 Aflatoxin at the rate of 20 ppm while Aflatoxin B2, G1 and G2 had not reported any occurrence on samples same results had been found with Sabzimandi sample. The rate of Aflatoxins on Sadar Rawalpindi samples had been determined B1 at the rate of 40 ppm while Alfatoxin B2, G1 and G2 had not showed any occurrence on samples.

Results shown in table (4.3) at the Raja Bazar B1 and G1 Aflatoxin at the rate of 40 ppm while Alfatoxin B2, and G2 had not showed any occurrence on sample. Results shown in table at the GLT Baseen B1 and G1 Aflatoxin at the rate of 10 ppm while Alfatoxin B2, and G2 had not showed any occurrence on samples.

Reason may be all plum stock in that shops and markets were well kept at optimum temperature and at less humidity. Because both factors play an important role in development of

pathogen or in growth of pathogen. Stock were stored in boxes so there is less chances of contamination by other products. They all have new stock of season which is harvested recently.

Results of Data Analysis:

In table 3 shows that collected samples from 12 different places and analysis was done by HPLC test and we have aflatoxins detected in six samples and apply statistics on the value. The (DF) degree of freedom is 3, some of square (SS) is 1089.58, Mean square (MS) is 363.194, Frequency (F) is 3.94 and level of significance is 0.0142 and then lastly, we find the grand means which is 3.958.

The after effects of the current review demonstrate that dried plum tests contained aflatoxins past the most extreme admissible constraint of (either mean worth of Aflatoxin or Sum Value fixed all out aflatoxins in dried natural products for human utilization utilizing HPLC detected AFB1, AFB2, AFG1 and AFG2 with mean value of 11.667, 0, 4.667 and 0 ppm respectively in dried Plum.

The event of Aflatoxin B1, B2, G1 and G2 was explored by HPLC in dried plum. Tests of the all-out 12 examples evaluated for the presence of mycotoxins, 6 examples were viewed as sure for AFB1 and AFG1 pollution with the mean scope of 3.95 ± 0.0142ppm. Be that as it may, 6 of the examples showed the rate of AFB1and AFG1 (Table 4.4). The aftereffects of the current review show that dried plum tests contained aflatoxin past the most extreme admissible constraint of 50 mg/g fixed aflatoxins in dried natural products for human utilization.

DISCUSSION

The world production of Plum in 2017 was 11,758,135 metric tons and its production in Pakistan around 49,800 tons of production in 2017-2018. Most of it comes from the provinces of Balochistan and Khyber Pakhtunkhwa. Kalat, Mardan, Nowshera, Peshawar, Pishin, Quetta, and Swat are among the top plum-producing regions in Pakistan. We conceived the current investigation to assess the mycoflora linked to aflatoxins in dried plump. Aspergillus flavus, Aspergillus parasiticus, and Aspergillus nomius are only a few of the fungus species that produce the polyketide pathway derivatives known as aflatoxins, which are found in many different types of food and feed (Smith *et al*., 2019). In this study, twenty-five samples (samples size) of dried plums from different markets were evaluated by applying the International Seed Testing association method (1985). In order to detect aflatoxins, The presence of aflatoxin B1, B2, G1, and G2 in dried plum was analyzed using high performance liquid chromatography. Mycotoxin testing on a total of 12 samples returned 6 positive results, with the average concentration of AFB1 and AFG1 in these samples coming in at 3.95 0.0142 ppm. However, AFB1 and AFG1 were found to be present in 6 of the samples (Tables 4-3). While in storage or waiting to be packaged, dried fruits and nuts are especially vulnerable to fungal contamination and aflatoxin buildup (Wu et al., 2018). According to the findings of the current investigation, dried plum samples had levels of aflatoxin higher than the European Commission's limit of 4 mg/g for total aflatoxins in dried fruits intended for human consumption. Dried fruits that have a high concentration of mycotoxins represent a health risk since they are a natural pollutant. Morton et alresearch .'s on aflatoxin contamination showed that dried plums and dried figs had the greatest likelihood for aflatoxin contamination. The levels of OTA in dried plums varied from 50 to 110 lg/kg, according to the results of an assessment for naturally occurring aflatoxin B1, B2, G1, G2, ochratoxin, and patulin

conducted on a variety of Egyptian dried fruits. These dried fruits tested negative for all other mycotoxins. In a similar vein, research by (Celik and Ozturk, 2000) found that plums dried on dirt and tarp and then treated with sulphur dioxide contained between 0.10 and 1.47 lg/kg of aflatoxin B1 and G1, whereas the corresponding values for untreated dried samples were between 0.35 and 1.27 lg/kg. Iranian researchers looked into the presence of two toxins, Aflatoxin and ochratoxin A, in dried plums using immunoaffinity column clean up and HPLC and found AFB1, AFB2, AFG1, and OTA with mean values of 0.88, 0.32, 0.20, and 2.83 ppm, respectively (Janati et al., 2010). Aflatoxin levels in dry fruits have been documented in several research (Zahra et al., 2019; Wang et al., 2018); these investigations come from all over the globe (Kang et al., 2010). All all, High Performance Liquid Chromatography found AFB1, AFB2, AFG1, and AFG2 in only 6 of 12 dried plum samples from Rawalpindi, Islamabad, and Gilgit, with mean values of 11.667, 0.00, and 4.667, respectively.

CONCLUSION

The level of contamination of dry plump with aflatoxins in Punjab and Gilgit Baltistan, Pakistan, was investigated in this study. Our finding revealed that dry plump from Rawalpindi and Gilgit Baltistan collected during study was contaminated with B1 and G1 followed by infection observed in other 6 samples of Aflatoxin detected during High Performance Liquid Chromatography, AFB1, AFB2, AFG1 and AFG2 with mean values 11.667, 0, 4.667, respectively. It is therefore recommended that the consumption of dry plumps must be paralleled by efficient measures for prevention and detoxification of Aflatoxin contamination for the provision of healthy and nutritious dry fruits.

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