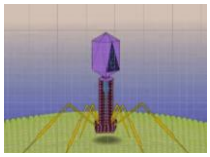

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ESTIMATION AND DETOXIFICATION OF AFLATOXINS IN OSTRICH FEED SAMPLES COLLECTED FROM LAHORE, PAKISTAN

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ABSTRACT: *Ostrich farming is an emerging agricultural business in Pakistan with high demands for meat, eggs, oil, feathers, and leather. However, the presence of aflatoxins may lead to health hazards to poultry and consumers depending on this industry. A study for aflatoxin contamination was conducted on ostrich feed samples collected from 10 localities of Lahore with an emphasis on storage and handling conditions. The samples were analyzed using thin liquid chromatography. The aflatoxin positive feed samples were then detoxified using 0.5, 1, and 1.5 g bud powder of Moringa oleifera, incubated for 7, 14, 21, and 28 days, and were analyzed again. Results revealed maximum contamination (60%) during summers with an average temperature of 30.88 ºC and minimum (20%) in winters with an average temperature of 17.08 ºC. The major contaminant that found was B1 Aflatoxin. Results were statistically analyzed by applying Paired T–Test which proved the effectiveness of M. oleifera ratio in detoxification of feed aflatoxins. On the basis of this study, it was concluded that M. oleifera buds are an efficient biological agent against aflatoxins in feed. In addition, control of temperature and improvement in storage conditions at the sites can reduce the risks of aflatoxicosis.*

Keyword: Aflatoxins; Chromatography; Feed; *Moringa oleifera*; Ostrich

INTRODUCTION

Aflatoxins (AFs) are a group of extremely poisonous mycotoxins produced by *Aspergillus spp.* which contaminate agricultural commodities (Dhakal and Sbar, 2020). These are hepatotoxic and carcinogenic mutagens that originate among cereals, oilseeds, spices, and nuts imposing immunotoxicity and teratogenicity in animals, birds, and humans (Iqbal et al., 2014). Humidand warm temperature increases contamination level (Ghorbanian et al., 2007). The period of storage influences the rate of toxin production by the fungus (Tajkarimi et al., 2014). Damaged grains exhibit higher levels of aflatoxins (Ozay et al., 2008). Out of the identified 20 types, the major ones include aflatoxin B1, B2, G1, and G2; metabolized to aflatoxin M1 and M2 (Boudra,2007) that form during the logarithm growth phase of the fungus and are named on the basis of fluorescence (Rawal, 2008). Consumption of affected animal-derived products such as meat, eggs, and milk cause human susceptibility to aflatoxins (Hayes, 1980). Aflatoxin exposure affects the 4.5 billion population of developing countries (Miklós, 2020). The high global exposure levels serve as a stark reminder that the preventive and mitigating measures put in place so far have not been adequate. Since the generation of aflatoxins by toxic molds is primarily reliant on environmental circumstances that are out of human control, their complete eradication from the world's food chains is impossible (Jallow, 2021) and ultimately affect the country's economy thereafter their prevention is of utmost importance (CAST, 2003).

Ostriches are important birds for livestock industries for the purpose of eggs and meat low in fat, cholesterol, and calories. Their skin and feathers are also used to manufacture leather, hats, and pillows (Brassó, 2020). There are 179 registered ostrich farms in Punjab, out of which nine occur in Lahore, having a total of 306 birds. The successful rearing of ostrich demands a proper diet balance helping in better growth and reproductive performance of the bird (Cooper, 2004). The present food and feed production systems that are animal or plant-derived, have been associated with various contaminants resulting from multiple sources and are declared unfit for human usage (Bilal, 2014). About 70% expense of animal production is on feed (Ajmal et al., 2022). Favorable weather and poor management in Pakistan feed mills

enhance aflatoxin contamination of poultry feed (Iheshiulor et al., 2011). The effect of aflatoxin depends on the age, sex, species, and nutritional status of the bird (Fareed et al., 2014). Therefore, a systematic examination and integrated aflatoxin control is needed to address the agricultural production and distribution chain, rather than adopting a few marginally effective technologies (Moral et al., 2020; El-Hack et al., 2020).

Biological control is an emerging approach for the degradation of toxins with no threats to health or food material and can significantly reduce 20-90% of infections (El-Hack et al., 2020, Waliyar et al., 2015). *Moringaoleifera* (L.) commonly called "the drumstick tree" is characterized as "miracle or wonder tree" because of its numerous nutritional, industrial, and pharmacological properties (Kunyanga et al., 2013). It is a valuable plant of the family Moringaceae; with biological and nutritional versatility as a fertilizer, bio-pesticide, medicine, feed, and seed production, because it is inexpensive and an environment-friendly species (El-Hack et al., 2018). Owing to the rising demands of moringa usage, the respective market is expected to rise up to 9.3% exceeding USD 6 billion by 2025 (Islam et al., 2021). Meal prepared from moringa leaf is a good feed supplement improving the performance, health, and growth of the animal/bird and is safe to use at an adequate level (Donkor et al., 2013). It increases the palatability, stability, processing, and shelf life of poultry and poultry products (Jung et al., 2010). Researchers around the globe conclude that *M. oleifera* meals could effectively be used a protein source for goats (Qwele et al., 2013), cattle (Mendieta-Araica et al., 2013), chicken (Wapi et al., 2013) and fish (Abdel-Latif et al., 2021). Despite intensive findings on biological means, the results are an initial step, and implementation is further required to reduce aflatoxins to acceptable limits without any toxic residues or loss in nutritive value while being economic and environment-friendly (Guo et al., 2021). The objectives of this study were to estimate contamination of aflatoxin in ostrich feed samples along with an assessment of different management practices at sampling and storage sites and to investigate the effect of *M. oleifera* bud powder for aflatoxin detoxification.

MATERIALS AND METHODS

Sampling Sites

A total of 560 samples of Ostrich feed were collected for experimentation. These were gathered from different captive sites (Lahore Zoo, Lahore Zoo Safari, and Jaloo Wildlife Park), commercial farms (Ajwa Farm near Thatatalwa and Ostrich Ranch Raiwind), feed manufacturing mill (Hi-Tech), breeding sites (Riphah International University) and markets (Tollinton market, Akbari mundi, and Chuburji market). These sampling sites are shown in the map (Fig. 1).

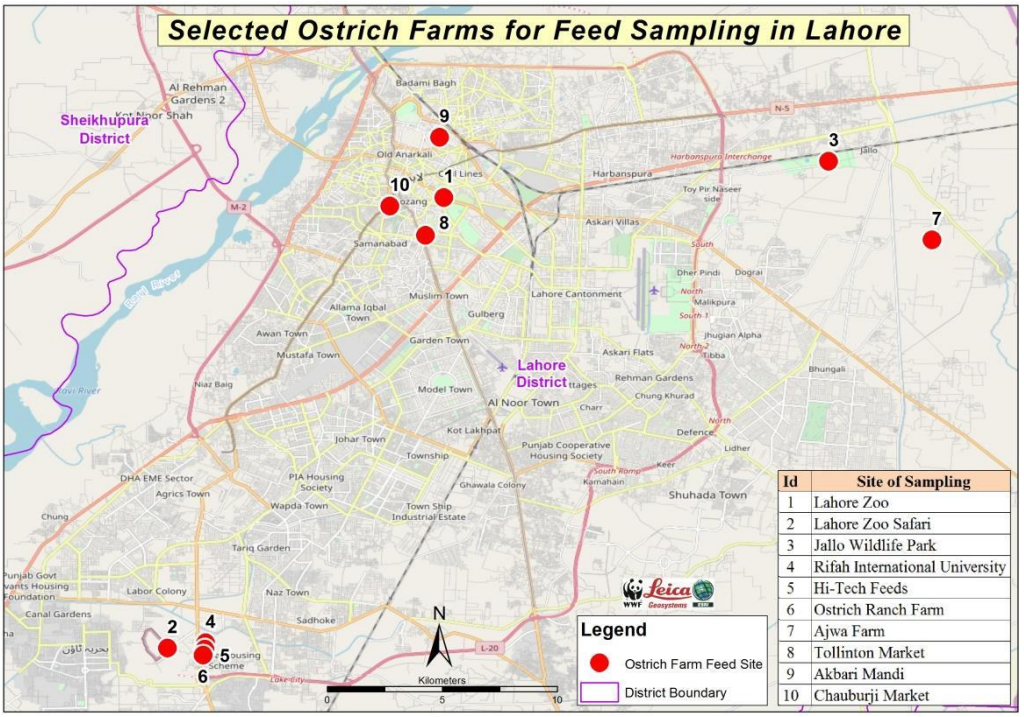


Fig. 1. Map of Lahore showing sampling sites

The study period remained continue for two seasons: October 2021 to February 2022 (Winter season) and May to September 2022 (Summer season). Grab sampling for feed was done weekly at aviaries and at an interval of 15 days at the depots owing to the purchase and storage duration of feed (total of 280 samples in one season). The samples collected at aviaries consisted of pre-feed (sample collected at the time of provision of feed to bird from purchased source) and post-feed (sample collected directly from the cage before cleaning) (Table 1).

Table 1: Sample Collection Details

S. ID	Location	No. of samples collected during each season		Total No. of samples collected
		Feed source	From cage	
1	Lahore Zoo	20	20	40
2	Jaloo Wildlife Park	20	20	40
3	Lahore Zoo Safari	20	20	40
4	Hi-Tech Mills	10	-	10
5	Refah University	20	20	40
6	Ostrich Ranch	20	20	40
7	Ajwa Farm	20	20	40
8	Tollinton Market	10	-	10
9	Akbari Mundi	10	-	10
10	Chauburji Market	10	-	10

A detailed questionnaire was designed for collecting information about Ostrich, its feed, management practices, handling, staff knowledge and practices, consumption, cleanliness, temperature, and humidity. The average temperature recorded during summers was 28.6-33.2°C ± 2 with a relative humidity of 70-76% ± 5 and 12.1-25.2 °C ± 2 during winters with a relative humidity of 65-66 % ± 5.

The experimental procedures were carried out at Pakistan Council for Scientific and Industrial Research Complex, Lahore for each sample separately. For validation of data, triplicate analysis was performed according to scientific protocols along with sterilization and disinfection of instruments. The AOAC (Association of Official Analytical Chemists) methods of aflatoxin analysis (AOAC,2016) were followed to investigate the aflatoxin contamination in collected ostrich feed samples.

Estimation of Aflatoxins

Thin liquid chromatography was used for estimation (Salisu et al, 2021). A 50g of feed sample was weighed and added into the conical flask, with celite, distilled water, and chloroform, covered with aluminum foil paper, and fixed on a wrist shaker for 30 minutes. Then 50 ml of filtrate was collected from each sample and dried on a hotplate. About 0.5 ml chloroform was poured into the dried extract for dilution and 25 µl was spotted by micro-syringe, 1 cm above the bottom, on TLC plate. Diethyl-ether was added in one tank as the first mobile phase while acetone and chloroform (1: 9) were taken in the other tank as the second mobile phase. After completing the spotting of all samples and standards the TLC plate was eluded in the first and second mobile phases one by one. It was removed, dried, and observed under UV light to note the results. The formula (Ellison and Williams, 2012) used for the determination of Aflatoxins was:

$$\text{AF content (µg/kg)} = \frac{S \times Y \times V}{W \times Z}$$

Where, S = Volume of aflatoxin standard for spotting (µl)

Y = Concentration of aflatoxin given in reference standard (µg/ml)

V = Volume of solution used to dissolve dried aflatoxin (µl)

W = Effective weight of original sample in extract (g)

Z = Volume of sample extract for spotting (µl)

Detoxification

For detoxification of aflatoxins buds of *Moringa oleifera* were identified, purchased from the market, spread on a sheet of paper, and air dried for two weeks. The samples were finely grounded to powder form and stored in a polythene bag at 4 ºC. Then three sets were prepared consisting of four flasks to be incubated for 7, 14, 21, and 28 days respectively with 50 g positive detected feed sample, water, chloroform, and *Moringa oleifera* bud powder in concentrations of 0.5, 1, and 1.5 g. TLC analysis was performed for each sample again and changes in aflatoxin levels were evaluated.

STATISTICAL ANALYSIS

The results were then statistically analyzed by applying Paired t-Test according to Steel et al. (1997).

RESULTS

Aflatoxin Analysis

The analysis revealed AFB to be the main contaminant in feed samples.

Aflatoxin G was not detected altogether. No significant differences were noted between pre-feed and post-feed. The results of winter samples revealed that 40 out of 280 were positive for Aflatoxin B1 from two locations mainly (Fig. 2).

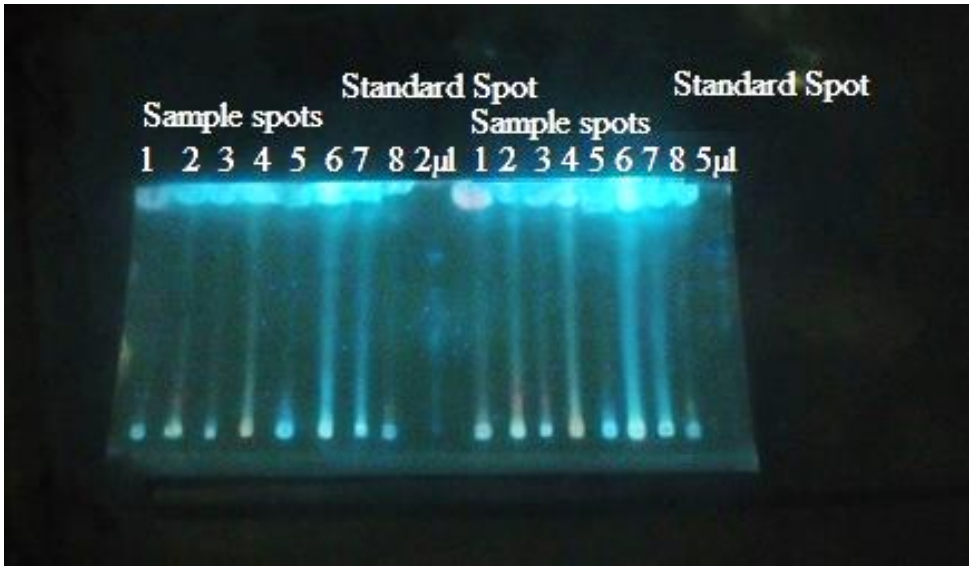


Fig. 2. A developed plate of winter samples observed under UV light for Aflatoxin detection

The mean values are represented in Table 2.

Table 2: Estimation of Aflatoxins in Ostrich Feed Samples Collected during Winter Season

S. ID	Area of Sampling	Aflatoxins				Total Aflatoxins	Status
		B1	B2	G1	G2		
1	Lahore Zoo	ND	ND	ND	ND	0	Fit
2	Jaloo Wildlife Park	ND	ND	ND	ND	0	Fit
3	Lahore Zoo Safari	1.98 ppb	ND	ND	ND	1.98 ppb	Contaminated
4	Hi-Tech Mills	ND	ND	ND	ND	0	Fit
5	Rephah University	ND	ND	ND	ND	0	Fit
6	Ostrich Ranch	ND	ND	ND	ND	0	Fit
7	Ajwa Farm	ND	ND	ND	ND	0	Fit

8	Tollinton Market	5.80 ppb	ND	ND	ND	5.80 ppb	Contaminated
9	Akbari Mundi	ND	ND	ND	ND	0	Fit
10	Chauburji Market	ND	ND	ND	ND	0	Fit

*ND – Not Detected

Out of 280 samples of the summer collection, 120 samples were found to be contaminated at six sampling sites (Figure 3).

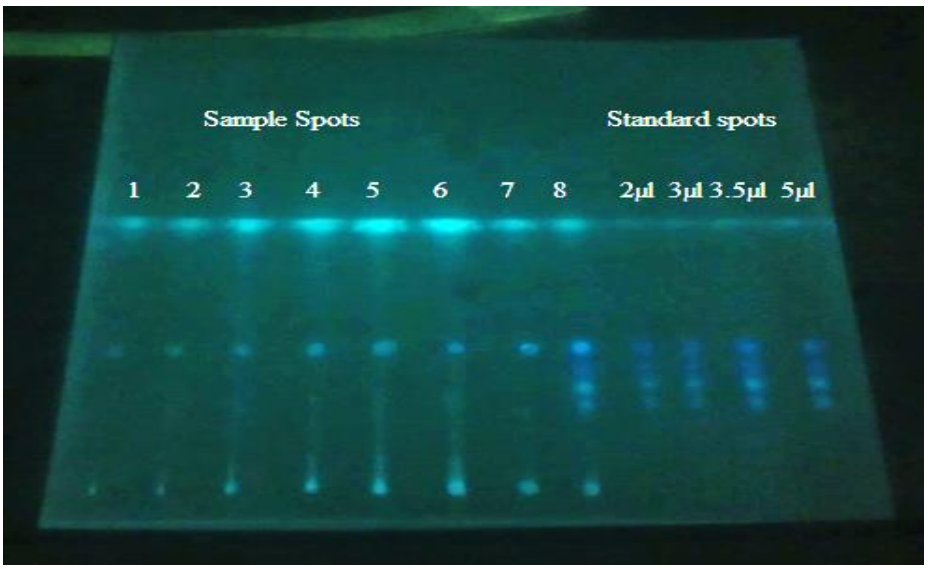


Fig. 3. A developed plate of summer samples observed under UV light for Aflatoxin detection

However, no health distress was recorded in any area. The average mean values shown in Table 3.

Table 3: Estimation of Aflatoxins in Ostrich Feed Samples collected during the Summer Season

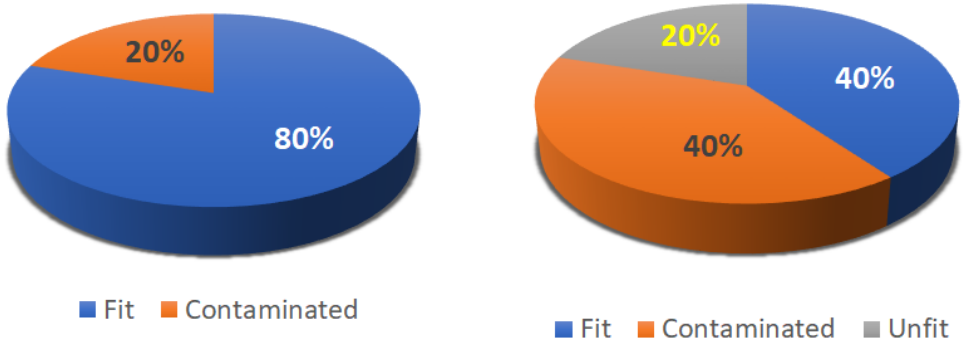
S. ID	Area of Sampling	Aflatoxins				Total Aflatoxins	Status
		B1	B2	G1	G2		
1	Lahore Zoo	ND	ND	ND	ND	0	Fit
2	Jaloo Wildlife Park	ND	ND	ND	ND	0	Fit
3	Lahore Zoo Safari	2.70 ppb	ND	ND	ND	2.70 ppb	Contaminated
4	Hi-Tech Mills	ND	ND	ND	ND	0	Fit
5	Refah University	38.79 ppb	ND	ND	ND	38.79 ppb	Contaminated

6	Ostrich Ranch	67.59 ppb	ND	ND	ND	67.59 ppb	Contaminated
7	Ajwa Farm	ND	ND	ND	ND	0	Fit
8	Tollinton Market	262.63 ppb	ND	ND	ND	262.63 ppb	Unfit
9	Akbari Mundi	135.88 ppb	ND	ND	ND	135.88 ppb	Unfit
10	Chauburji Market	14.66 ppb	1 ppb	ND	ND	15.66 ppb	Contaminated

ND – Not Detected

Assessment of Seasonal Variation and Storage Practices

The mean temperature recorded by thermometer (KI and BNT [DT-2]) during both collections was 17.08-30.88 ^{o}C with the lowest and highest readings of 12.1-33.2^{o}C from winter to summer. These results of AF contamination reveal annual fluctuation that is brought by weather-related causes (temperature and humidity) (Fig. 4).



(a) (b)

Fig. 4. (a) Percentage of aflatoxin contamination in ostrich feed samples during winters. (b) Percentage of aflatoxin contamination in ostrich feed samples during summers

The data obtained showed that storage practices directly influenced contamination by aflatoxins in feed samples especially at farm and market areas where the feed was stored in bulk quantity for longer time periods at goddam with lack of proper ventilation (Table 4).

Table 4: Relationship of Management Facilities at Sampling Sites with Contamination of Aflatoxins in Feed Samples

S. ID	Site	No. of birds	Storage condition	Storage practice	Storage container	Storage time (months)	Cleaning / day	Feeding / day	Total Aflatoxins
1	Lahore Zoo	2	Open air	Proper	Jute bags	--	Twice	Twice	0 - 0
2	Jaloo Wildlife Park	5	Open air	Proper	Plastic bags	--	Thrice	Twice	0 - 0
3	Lahore Zoo Safari	4	Open air	Proper	Plastic bags	--	Once	Thrice	1.9 - 2.7
4	Hi-Tech Mills	--	AC rooms	Proper	Plastic bags	--	Once	--	0 - 0
5	Riphah University	7	Godam	Poor	Plastic bags	3-5	Twice	Twice	0 - 38.7
6	Ostrich Ranch	50	Godam	Poor	Plastic bags	2-3	Twice	Twice	0 - 67.5
7	Ajwa Farm	15	Godam	Fine	Plastic bags	2-4	Twice	Twice	0 - 0
8	Tollinton Market	--	Bulk stores	Bad	Plastic bags	1-2	Once	--	5.8 - 262.6
9	Akbari Mundi	--	Bulk stores	Bad	Jute bags	1-2	Once	--	0 - 135.8
10	Chauburji Market	--	Bulk stores	Fine	Plastic bags	1-2	Once	--	0 - 15.6

Detoxification study

All three concentrations of *M. oleifera* bud powder used for detoxification of contaminated ostrich feed successfully degraded the aflatoxins (Table 5), especially the quantity of 1.5 g provided the finest result by removal of AF with 100% accuracy during the incubation period.

Table 5: Degradation of aflatoxins in feed samples using bud powder of *Moringa oleifera* incubated for four weeks

S. ID	Sampling site	Aflatoxin concentration (ppb)	Incubation period (days)	Aflatoxin concentration (ppb) after degradation by *Moringa oleifera* bud powder (g)		
				0.5	1	1.5
1	Tollinton Market	262.63	7	230.62	198.49	153.91
			14	171.80	133.07	51.12
			21	111.59	66.44	2.73
			28	79.95	9.97	ND
2	Akbari Mundi	135.88	7	111.62	97.80	87.85
			14	75.75	63.23	38.15
			21	49.83	24.87	ND
			28	14.85	ND	ND
3	Ostrich Ranch	67.59	7	58.65	49.25	29.94
			14	49.83	27.93	ND
			21	28.35	16.61	ND
			28	19.15	ND	ND
4	Refah University	38.79	7	32.49	29.59	ND
			14	24.72	19.03	ND
			21	14.49	8.70	ND
			28	3.49	ND	ND
5	Chauburji Market	14.66	7	11.66	9.84	ND
			14	7.78	4.59	ND
			21	3.18	ND	ND
			28	ND	ND	ND

DISCUSSION

Aflatoxin contamination of animal feed is a major persistent worldwide problem. It reduces the effectiveness of the feed and increases susceptibility in livestock, leading to mortality, feed waste, and production expenses (Giuberti et al., 2021). The livestock sector is the cheapest available meat and egg source of animal protein for the population. This sector has suffered substantial economic losses in Pakistan for quite a long time due to AF contamination linked to persistent

humidity and the rainy season. The mean AFB_1 levels in feed analyzed in Pakistan are reported in a range of 3.04-214.9 ppb as described by Ajmal et al. (2016). The results obtained during this study also represent a range of 1.98-262.63 ppb from winter to summer. These are consistent with the range of source materials reported in Biomin's yearly global mycotoxin surveys (Biomin Annual Mycotoxin Surveys, 2020). Alam et al. (2016) also reported similar findings from 216 samples of chicken feed collected in the Swat, Peshawar, and D. I. Khan districts of KPK, Pakistan, throughout the summer, winter, fall, and spring with high contamination levels of AFB1, AFB2, AFG1, and AFG2. The contaminated samples of this study below the Maximum Tolerance Limit of 100 set by the United States Food and Drug Administration for livestock were declared fit for usage while 40 summer samples above the permitted ratio were declared unfit. Pakistan's warm, humid climate is ideal for the invasion of mycoflora. Summer samples contained the highest quantities than those recorded during winter in the same way described by Alam et al. (2016). This difference may be the result of the feed being produced under various feeding practices, agroecological circumstances, climatic variables, and feed storage conditions. Due to these reasons, a variety of feed ingredients are prone to mold formation in Pakistan, which increases the risk of AF contamination. High incidence may ultimately lead to aflatoxicosis in poultry. Hence the livestock industries experience significant economic losses as a result of the detrimental effects of contaminated animal feed (Oluwatobi et al., 2022). Crops are vulnerable to AF contamination as a result of the poor agronomic and storage practices used by farmers and processors that were found during the investigation. These are in line with Toma et al. (2019) who reported that only 55% of the 234 survey participants had some awareness about aflatoxin, while the remaining 44.1%, did not have any knowledge regarding to it.

Ajmal et al. (2022) reviews that the season has a significant impact on fungal infestation A hot monsoon season, which normally lasts from June to September, and persistent relative humidity have both been connected to changes in feed AF contamination in Pakistan. Chauhan et al. (2016), which found the highest AFB1 levels from June to November and the lowest from

December to May. These results were in consistent with those of Hussain et al. (2023), who recorded high aflatoxin contamination in summer than winter season in the animal feed. Climate, temperature, and precipitation patterns enhance the production of mycotoxin and combined with untimely harvest time, improper drying, and poor storage can cause an economic loss of up to $1.7 billion annually. Both pre-harvest handling and post-harvest handling require the management of aflatoxin in order to minimize the chance of occurrence as apparent in the study. Enhancing crops' resistance to fungal infections or avoiding the generation of aflatoxins by fungal invasion are the two main long-term, sustainable solutions to control pre-harvest aflatoxin contamination (Awuchi, 2020).

Better storage strategies were adopted at commercial sites as they particularly aim for the recreational conservation of the bird with the purpose of health, breeding, education, and research. In market areas, the feed ingredients were open in contact with the environment and hygiene facilities are poor. At the farm areas storage rooms were quite poorly managed. The results were similar to Khan (1994) examination of 331 samples of cottonseed from farms, feed mills, and godowns and retail stores in Karachi, Pakistan, and discovered that 70% of the samples tested positive for AFB1, with an average value of 155.7 lg/kg and a range of 3-1,629 lg/kg. These areas purposely raise ostrich for meat and eggs and any chance of illness in the bird can directly threaten the consumer's health and life. Tadele et al. (2023)stated that feed stored for more than 6 months was 5.8 times more likely to have AF contamination levels above the European Union limit as observed in summer samples of farm and market sites. Toma et al. (2019) described that farmers without animal feed storage facilities were five times more likely to have AF contamination above the EU allowed level than those who did. Mold growth and the development of aflatoxins may be facilitated by prolonged exposure to high humidity, insufficient ventilation and temperatures, plastic bag storage indoors, long-term storage, and a lack of higher-ventilated storage platforms. Hence, integrated and harmonized monitoring strategies and regulation limits responding to the presence of aflatoxins along the entire food chain should be developed and implemented

globally (Zafar et al., 2001, Valencia-Quintana et al., 2020).The scope should include all stages of primary production, from agricultural production to the output from livestock farms, passing through the feed industries; in the agricultural sector, through the adoption by farmers along with the development of new technologies for the prevention and control of aflatoxins in their crops, during harvest and in storage (Noemi et al., 2021).

Aflatoxin mitigation is key to food safety and nutrition along the food chains (Pickova et al., 2021). *M. oleifera* exhibits promising potential due to its prominent antidiabetic, antioxidant, anti-inflammatory, and other bioactive properties to create food composition, enhance health and boost nutritional profile (Valencia-Quintana et al., 2021). The use of MO bud powder for detoxification of aflatoxins proved fruitful and coincides with the findings of Sanni et al. (2021) on the evaluation and efficiency of moringa nutrition on broilers which enhanced their feed intake weight gain and growth rate. Estimating the total cost of around 500 g of *M.oleifera* buds equal to 50 PKR. This means that the Ostrich feed handlers can add 1½ kg (1500 g) *M.oleifera* buds or bud powder in a sack of 50 kg feed (50,000 g) to combat against AFs costing about 5000 PKR only. Therefore, a little quantity may prove a cheap and effective source of aflatoxin detoxification in feed products that will also improve the palatability, stability, processing, and shelf life of poultry and poultry products as stated by Shabeer et al. (2022). However, more research is required for a better understanding of the molecular mechanisms related to their functional properties (Noemi et al., 2021).

CONCLUSION

This study provides the information that highest contamination in summer samples were found as compared to winter season. Moreover, the stuy gives a valuable insight about the prevalence and risk of aflatoxins that are affecting the quality of feed products and if left unchecked due to poor storage practices could cause serious distress in the ostrich leading to loss of farming practices. Moringa buds may be mixed in feed samples to enhance the nutritional value and reduce the risk of product contamination. Further research, monitoring, and implementation of detoxification strategies will lead to a sustainable poultry sector.

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