Full Length Article

ASSESSMENT OF THE OCCURRENCE OF MYCOTOXINS IN LIVESTOCK FEEDS AND FEED INGREDIENTS

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ABSTRACT

Mycotoxins are carcinogenic, mutagenic and teratogenic toxic metabolites that are known to *cause detrimental effect on the health of animals consuming its contaminated feed. The objective of this study was to study the seasonal occurrence of mycotoxins, aflatoxins (B1, B2, G1 and G2), citrinin, ochratoxin A and T-2 toxin in feed and feed ingredients submitted to Pharmacovigilance Laboratory for Animal Feed and Food Safety (PLAFFS). A total of 412 samples which included feed (289) and feed ingredients (123) received during 2019-2020 were analyzed using thin layer chromatographic method. Among the 249 positive samples, the incidence of aflatoxin B1, aflatoxin B2, Aflatoxin G1 &G2, citrinin and ochratoxin A were 85.54%, 14.06%, 1.2%, 20.08%, 3.21% and 2.41% respectively and the concentration of these mycotoxins ranged between 1.14 to 899.78 µg kg^{-1}. Only few samples were contaminated with aflatoxin G1 & G2, Ochratoxin A and T-2 toxin. Seasons had an influence on the mycotoxin contamination and the highest incidence was detected during winter, whereas, Citrinin and T-2 toxin were maximum at monsoon and summer respectively. Regular monitoring of mycotoxin contamination in animal feed is essential to prevent the entry of mycotoxins in the food chain.*

Keywords: Feed and feed ingredients, Incidence, Mycotoxins, TLC.

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INTRODUCTION

Mycotoxins are secondary metabolites secreted by filamentous fungi mostly belonging to the genus *Apergillus*, *Penicillium* and *Fusarium*. They have been shown to be associated with animal feed and human food products and these toxins are known to be carcinogenic, mutagenic, teratogenic and

toxic (Creppy, 2002; Yiannikouris and Jouany, 2002). They are simple heterocyclic rings with a total molecular weight of >500 Da, and are not immunogenic (Souza *et al.,* 2013). They are found in the field before harvest, post-harvest or during processing, storage and feeding; adversely affecting the animal feed quality (Sforza *et al.,* 2006).

Fungi that can produce mycotoxins grow on numerous food and animal feeds, cereals, millets, pulses, dried fruits, nuts and spices. Most of the mycotoxins are chemically stable and cannot be decomposed or broken down by digestion, and hence survives the food and feed processing (Rai *et al.,* 2012). The most common mycotoxins detrimental to human health and livestock are aflatoxins, citrinin, ochratoxin, T2 toxin, patulin, fumonisins, zearalenone and nivalenol/ deoxynivalenol (DON).

Mycotoxins cause various types of adverse effects (mycotoxicosis) such as reduced productivity, increased disease incidence, chronic damage of vital organs and decreased reproductive performance and pose serious health threat to humans, livestock and crops to result in illness as well as economic loss (Zain, 2011). The degree of these adverse effects is determined by the concentration of toxin, duration of exposure and the health and age of the individual. However, it also affects the agribusiness in both developing and developed countries by impeding the export of agricultural commodities as well as reduces the production of livestock and crop farming (Leung *et al.,* 2006). There are more than 400 naturally occurring mycotoxins, however, only a few of them have been extensively studied (Whitlow and Hagler, 2005). The data on season wise occurrence of various mycotoxins in Tamil Nadu and other neighbouring states were not available. Hence, we have analysed the occurrence of various mycotoxins in feed and feed ingredients that were submitted to Pharmacovigilance Laboratory for Animal Feed and Food Safety (PLAFFS) collected from different regions of Tamil Nadu and other States over a period of 2 years.

MATERIALS AND METHODS

The livestock feeds and feed ingredients received at PLAFFS, Directorate of Centre for Animal Health Studies, Tamil Nadu Veterinary and Animal Sciences University, Chennai during the year 2019 and 2020 were utilized for the present study. A total of 412 livestock feed and feed ingredients were analysed for the level of contamination with different mycotoxins. From the samples, 25 g of finely ground samples were used for mycotoxins estimation as per AOAC method by TLC and quantified with respective reference standards.

Extraction of Aflatoxins

Aflatoxins were extracted by taking 25 g of ground sample in a 250 ml bottle to which 1.5 g of NaCl, 106 ml of acetone and 19 ml of distilled water were added. The mixture was shaken for 30 min. at 200 rpm in an orbital shaker and filtered (with Whatman paper No.1). From this filtrate, 75 ml was transferred into a 250 ml beaker containing 0.33g of activated cupric carbonate ($CuCO_3$), 85 ml of 0.2M NaOH and 15 ml of 0.4M $FeCl_3$, mixed thoroughly, the contents allowed to settle for 10 min. at room temperature and

the supernatant filtered (with Whatman paper No.1). From this filtrate, 100 ml was transferred into a 250 ml separating funnel containing 100 ml of 0.03% Sulphuric acid (H_2SO_4) and 25 ml of chloroform ($CHCl_3$). The mixture in the separating funnel was shaken vigorously and allowed to settle in the stand to separate in to two layers of the mixture. The lower part, chloroform layer was collected into another 100 ml separating funnel containing 40 ml of 0.02M potassium hydroxide (KOH) and 1% potassium chloride (KCl) solution. The resultant organic extract was passed through a sodium sulfate (Na_2SO_4) bed and collected in a 50 ml beaker and concentrated in a hot plate under the Fume hood. Finally the dried extract was reconstituted with 200 µl of $CHCl_3$ and used for aflatoxins analysis by TLC method.

Extraction of multi-mycotoxins

Multi-mycotoxins extraction was carried out by taking 25 g of ground sample in a 250 ml bottle and mixed with 1.5 g of NaCl, 88 ml of acetonitrile, 2 ml of 20% H_2SO_4 and 10 ml of 5N HCl and 4% KCl solution. The mixture was shaken for 30 min. at 200 rpm in an orbital shaker and filtered (with Whatman paper No.1). Then 50 ml of the filtrate was transferred into a 250 ml separating funnel containing 50 ml of hexane and 50 ml of distilled water. The mixture in the separating funnel was shaken vigorously and allowed to settle in the stand to separate in to two layers of the mixture. This step was repeated with 50 ml hexane alone and the lower part was collected into a separating funnel. Then 20 ml of chloroform ($CHCl_3$) was added and mixed thoroughly. The resultant organic extract was passed through a Na_2SO_4 bed, collected in a 50 ml beaker and concentrated in a hot plate under the Fume hood. Finally the dried extract was reconstituted with 0.4 ml of benzene acetonitrile and analysed for multi-mycotoxins by TLC.

Preparation of standards

The certified reference material (CRM) of the mycotoxin standards were purchased from M/s. Himedia Pvt. Ltd. The standards were prepared in an amber coloured volumetric flask by dissolving the aflatoxin B_1, B_2, G_1 and G_2 in Benzene and Acetonitrile (98:2 ratio), Citrinin in Chloroform, Ochratoxin A in Benzene and Acetic acid (99:1 ratio) and T-2 toxin in Chloroform, labelled and stored as a main stock. From this, the working stock was prepared by suitable dilution and used for the analysis. The vials containing stock solutions were stored at refrigerated condition until use.

Determination of aflatoxins by TLC

From the reconstituted aflatoxins residue, 5 µl was spotted on a 0.5 mm thickness silica gel plate along with different volumes of respected reference standard. The plate was developed in 9:1 ratio of acetone and chloroform. Finally the plate was air-dried and observed under long wave UV lamp in a viewing chamber. The fluorescence intensities of aflatoxins in sample spot were compared with respective standard spots. Standard was also used to compare the colour and retention factor (Rf) value of unknown samples and the concentration was estimated.

Resolution of mycotoxins by TLC

The mycotoxins standards were resolved in a pre-coated silica gel 60 F254

(0.20 mm thickness), (5 x 10 cm, length) plates and the Rf-values were determined. The mobile phase systems used for developing the plates have been given in the Table 1.

Estimation of mycotoxins by TLC

All the mycotoxins were estimated by visual method. The extract of mycotoxins sample was spotted besides the respective standards, namely, aflatoxin B_1, aflatoxin B_2, aflatoxin G_1, aflatoxin G_2, citrinin, ocharatoxin A and T-2 toxin on a pre-coated TLC silica plate by using micro-syringe. The chemical structure of aflatoxin B_1, aflatoxin B_2, aflatoxin G_1, aflatoxin G_2, citrinin, ocharatoxin A and T-2 toxin was given in Fig.1. Based on the mycotoxins, the plates were developed with specific mobile phase system (Table 1). The plate was allowed to dry on a hot-plate and then examined in the UV-cabinet at 254 nm. Finally the intensity of the sample spots was compared with their respective standard mycotoxins and the concentration was calculated according to the following equation:

$$\frac{\text{Volume of standard (µl) X Concentration of standard (µg/ ml) X Final dilution}}{\text{Volume of sample (µl) X Effective weight of sample}}$$

The spiked samples were analysed in triplicate for estimating the recovery percentage.

RESULTS AND DISCUSSION

The average recovery percentage of aflatoxin B_1, aflatoxin B_2, aflatoxin G_1, aflatoxin G_2, citrinin, ocharatoxin A and T-2 toxin obtained from spiked feed samples were 96.08, 84.92, 95.34, 94.44, 94.68, 94.46 and 80 percent respectively; while, it was 89.56, 95.14, 98.66, 94.44, 86.56, 94.8 and 82.1 percent respectively from feed ingredients . The highest recovery rate was found in feed ingredients indicating that the sample matrix have an important role in the recovery of mycotoxins. The LOD was 5 µg kg^{-1} for

Table 1. Solvent system used for resolving the different mycotoxins

Mycotoxins	Pre-spray	Developing solvent	Spray
Aflatoxin	-	Chloroform : Acetone (9 : 1)	20% Sulphuric acid in methanol
Citrinin	10% Oxalic acid in methanol	Toluene : Ethyl acetate : Formic acid (6 : 3 : 1)	Ammonia vapour
Ochratoxin	10% Oxalic acid in methanol	Toluene : Ethyl acetate : Formic acid (6 : 3 : 1)	Ammonia vapour
T2 toxin	-	Chloroform : Acetone (9 : 1)	1.7% Chloroform

aflatoxin B_1 and aflatoxin B_2, 10 µg kg^{-1} for aflatoxin G_1 and aflatoxin G_2, 40 µg kg^{-1} for citrinin and Ochratoxin A and 100 µg kg^{-1} for T-2 toxin. Similar study was conducted in animal feeds (Marijana and Borka, 2006), spices (EL-Kady *et al*., 1995), pistachio nuts (Cheraghali *et al.,* 2007) and a traditional Turkish food, helva (Var *et al*., 2007).

Occurrence of mycotoxins in animal feed and feed ingredients

In both feed and feed ingredients samples (n = 412), 7 major mycotoxins were found (Table 2 and Fig. 2) and the most prevalent mycotoxin was aflatoxin B_1 (incidence, 52.43; range, 1.14-717.03 µg kg^{-1}; mean, 22.23 µg kg^{-1}). In total, 216 feed and feed ingredients samples were contaminated with aflatoxin B_1, 53 samples with citrinin, 38 samples with aflatoxin B_2, 8 samples with Ochratoxin A, 6 samples with T-2 toxin, 4 samples with aflatoxin G_1 and 3 samples with aflatoxin G_2. In feeds, 62.63% (n = 181) samples were contaminated with aflatoxin B_1, whereas 28.46% of feed ingredients samples (n = 35) were contaminated with aflatoxin B_1. It was observed that the maximum level of aflatoxin B_1 in feed was much higher than that detected in feed ingredients. Also, none of the feed ingredients were contaminated with aflatoxin G_1, aflatoxin G_2 and T-2 toxin. The incidence, minimum, maximum and mean values have been represented in Table 3 and 4.

The increased incidence of co-occurrence of multiple mycotoxins in animal feed and food products is a rising health concern due to the exposure to multiple fungal growths, which might exert greater toxicity than exposure to single mycotoxin. The co-occurrence of mycotoxins for feed and feed ingredients has been represented in Table 3. Out of contaminated samples (n = 249), the co-occurrence of aflatoxin B_1 and B_2 (11.65%) in the samples was higher as compared to other mycotoxins co-occurrence and the decreasing order of the co-occurrence of various mycotoxins is as follows, aflatoxin B_1 with citrinin (8.03%), aflatoxin B_1, aflatoxin B_2 and citrinin (1.61%), 1.2% each with aflatoxin B_1 and G_1, and aflatoxin B_1 and Ochratoxin A, 0.8% each with aflatoxin B_1 and G_2, aflatoxin B_1 and T-2 toxin and aflatoxin B_1, B_2 and G_2 (0.4%). Aflatoxin B_1 was co-occurring with other six other mycotoxins which show that it is the most common and predominantly occurring aflatoxin in both feed and feed ingredients. Ibanez-Vea *et al.,* 2011; Montes *et al.,* 2012; Iqbal *et al.,* 2014; Marie *et al.,* 2016; Carla *et al.,* 2018 and Palumbo *et al.,* 2020 reported the co-occurrence of aflatoxin with ochratoxin A and zearalenone; aflatoxin with DON and zearalenone; aflatoxin with ochratoxin A, DON, fumonisin and zearalenone in cereals, although they did not present data of co-occurrence. These multiple toxins can have additive, antagonist or synergic effects (Prosperini *et al.,* 2014; Ruiz *et al.,* 2011).

Climatic condition may contribute the key factor in driving the toxigenic fungi community and mycotoxin contamination levels at pre and post-harvest. Perrone *et al.* (2020) reviewed the effects of various climatic factors such as temperature, precipitation and atmospheric CO2 concentration on mycotoxins contamination level. The present study results revealed that the occurrence and mean concentrations of mycotoxins

Table 2. Occurrence level of mycotoxins in the feed and feed ingredients samples analyzed in the study

Mycotoxin	Contamination incidence [a]	Concentration (µg/kg)		
		Min	Max	Mean
AFB1	52.43	1.14	717.03	22.23
AFB2	9.22	2.12	119	17.8
AFG1	0.97	9.3	10	9.66
AFG2	0.73	12	15	14
Citrinin	12.86	9.8	246.12	42.46
Ochratoxin A	1.94	21	141.23	69
T2 Toxin	1.46	30	899.78	359.88

[a] Expressed in percentage

Table 3. Co-occurrence of mycotoxins detected in the feed and feed ingredients analyzed in this study

Mycotoxins combination[1]	Feed	Feed ingredients
$AFB_1 + AFB_2$	10%	18.37%
$AFB_1 + AFG_1$	1%	2.04%
$AFB_1 + AFG_2$	1%	n.d
AFB_1 + CIT	9%	4.08%
AFB_1 + OTA	1%	2.04%
AFB_1 + T-2	1%	n.d
$AFB_1 + AFB_2 + G_2$	0.5%	n.d
$AFB_1 + AFB_2$ + CIT	2%	n.d
CIT + OTA	n.d	6.12%

[1] AFB_1–Aflatoxin B_1 ; AFB_2–Aflatoxin B_2; AFG_1–Aflatoxin G_1 ; AFG_2–Aflatoxin G_2 ; CIT- Citrinin; OTA-Ochratoxin A; T-2-T-2 toxincccc

n.d - not detected

Table 4. Mycotoxins occurrence in feed and feed ingredients irrespective of the year

Metabolite	Feed (n^a=289 ; n^b=7)				Feed ingredients (n^a=123 ; n^b=5)			
	Incidence [c]	Concentration (µg/kg)			Incidence [c]	Concentration (µg/kg)		
		Min	Max	Mean		Min	Max	Mean
AFB1	62.63	2.4	717	26.61	28.46	2.56	126.02	19.85
AFB2	8.65	4.2	119	22.01	8.94	2.56	23.88	8.69
AFG1	0.69	10	10	9.77	0.81	9.32	9.32	9.32
AFG2	1.04	12	15	14	n.d	n.d	n.d	n.d
Citrinin	13.84	12.3	246.1	45.31	11.38	9.8	126.04	32.74
Ochratoxin A	0.69	21.2	80	50.5	4.88	27.28	141.23	75.17
T2 Toxin	2.08	30	899.8	359.88	n.d	n.d	n.d	n.d

n.d-not detected

[a] Number of samples analyzed

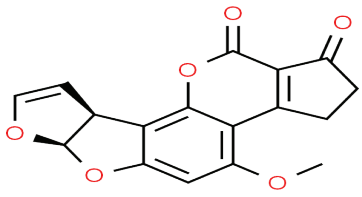
[b] Number of metabolites detected

[c] Incidence of contamination expressed in percentage

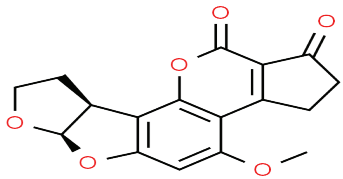
varied between seasons (Fig. 3). Several factors such as moisture content, temperature, pre and post harvest practices and storage conditions may influence these variations in the mycotoxins contamination levels in feed and feed ingredients. The seasonal variation of mycotoxins incidence was observed in the current study, namely, the incidences of aflatoxins (19.44 µg kg^{-1}) and ochratoxin A (80 µg kg^{-1}) during winter of the year 2019 was higher as compared to monsoon (21 µg kg^{-1}) and no occurrence during summer. However, the mean value of citrinin (42.25 µg kg^{-1}) and T-2 (824.65 µg kg^{-1}) toxin incidence was higher during monsoon and summer season respectively. Further, seasonal variation in the level of mycotoxin contamination might be due to the variation in the fungal community as the co-inhabiting mycoflora may have the antagonistic or synergistic effects (Ismail *et al.,* 2017). In the year 2020, the incidence of mycotoxins contamination could not be correlated with the seasons possibly be due to the delay in the receiving of samples from one season to another (due to the COVID-19 pandemic). Globally the prevalence and seasonal variation of mycotoxins incidence were reported in cow feed (Rozhin *et al*., 2015), animal and poultry feeds (Abdou *et al.,* 2017) and maize crop (Leggieri *et al.,* 2020).

CONCLUSION

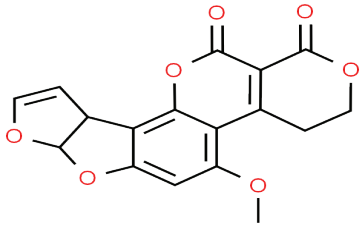
Mycotoxins in animal feed and feed ingredients are contaminants produced by toxigenic fungi during improper storage and



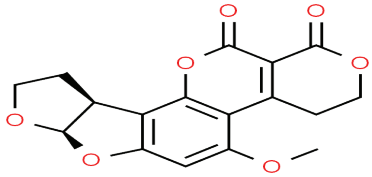
Aflatoxin B1



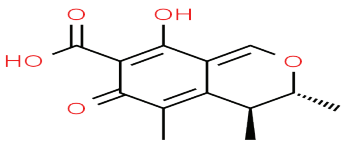
Aflatoxin B2



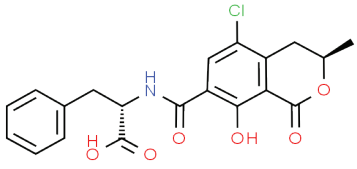
Aflatoxin G1



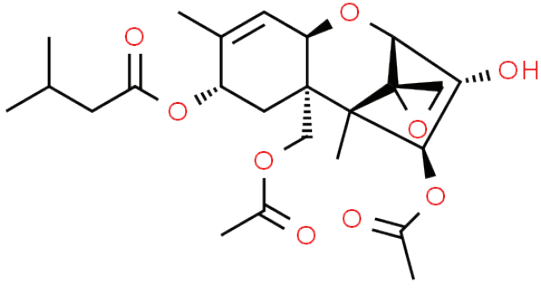
Aflatoxin G2



Citrinin



Ochratoxin A



T-2 toxin

Fig. 1. Chemical structure of aflatoxin B (AFB1 and AFB2), aflatoxin G (AFG1 and AFG2), Citrinin, Ochratoxin A and T-2 toxin (source: Chemspider).

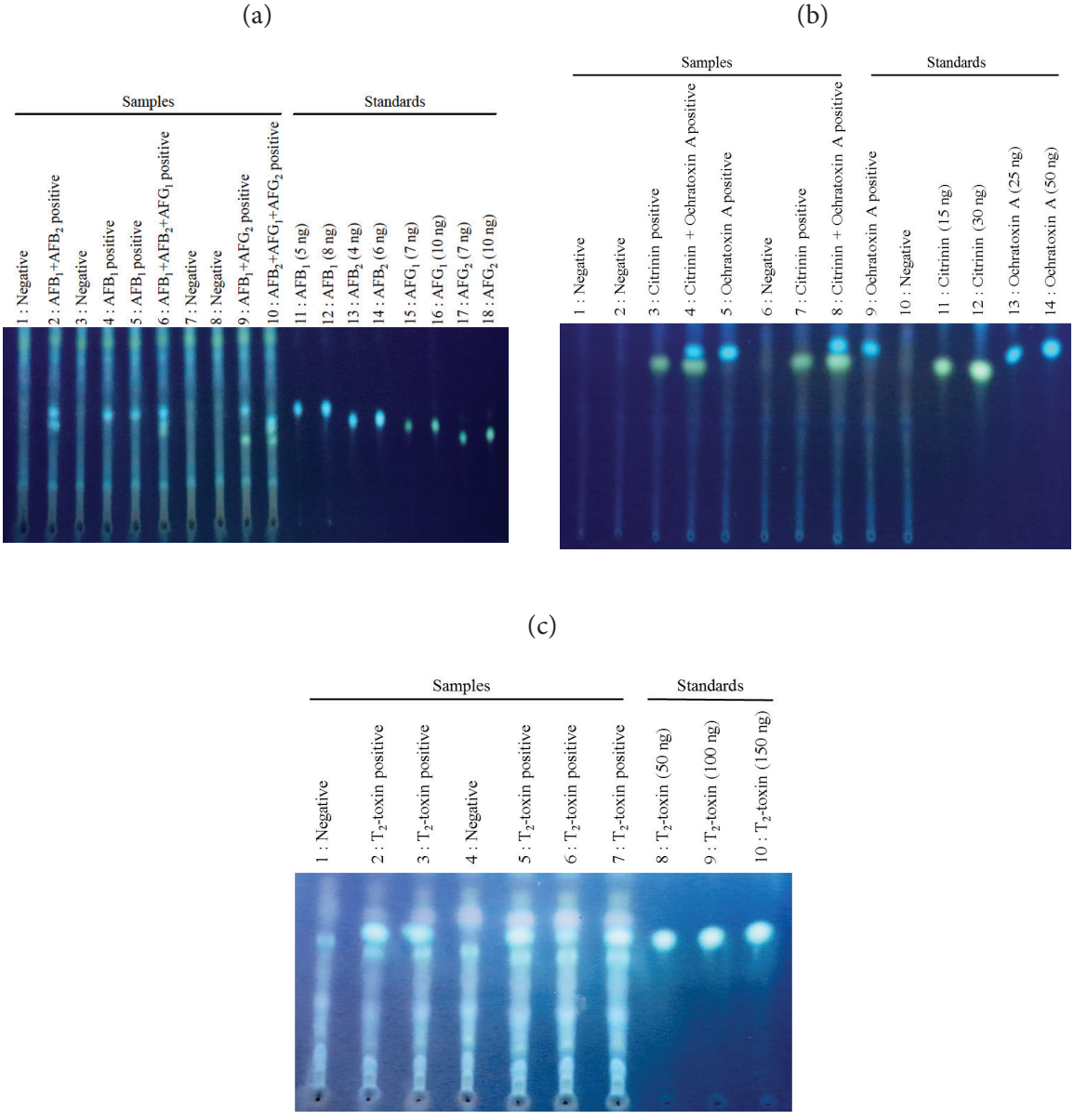


Fig. 2. Profile of Mycotoxin spots on thin layer chromatography. (a) aflatoxin B (AFB_1 and AFB_2) and G (AFG_1 and AFG_2); (b) Citrinin and Ochratoxin A; (c) T_2-toxin with various concentration of standards.

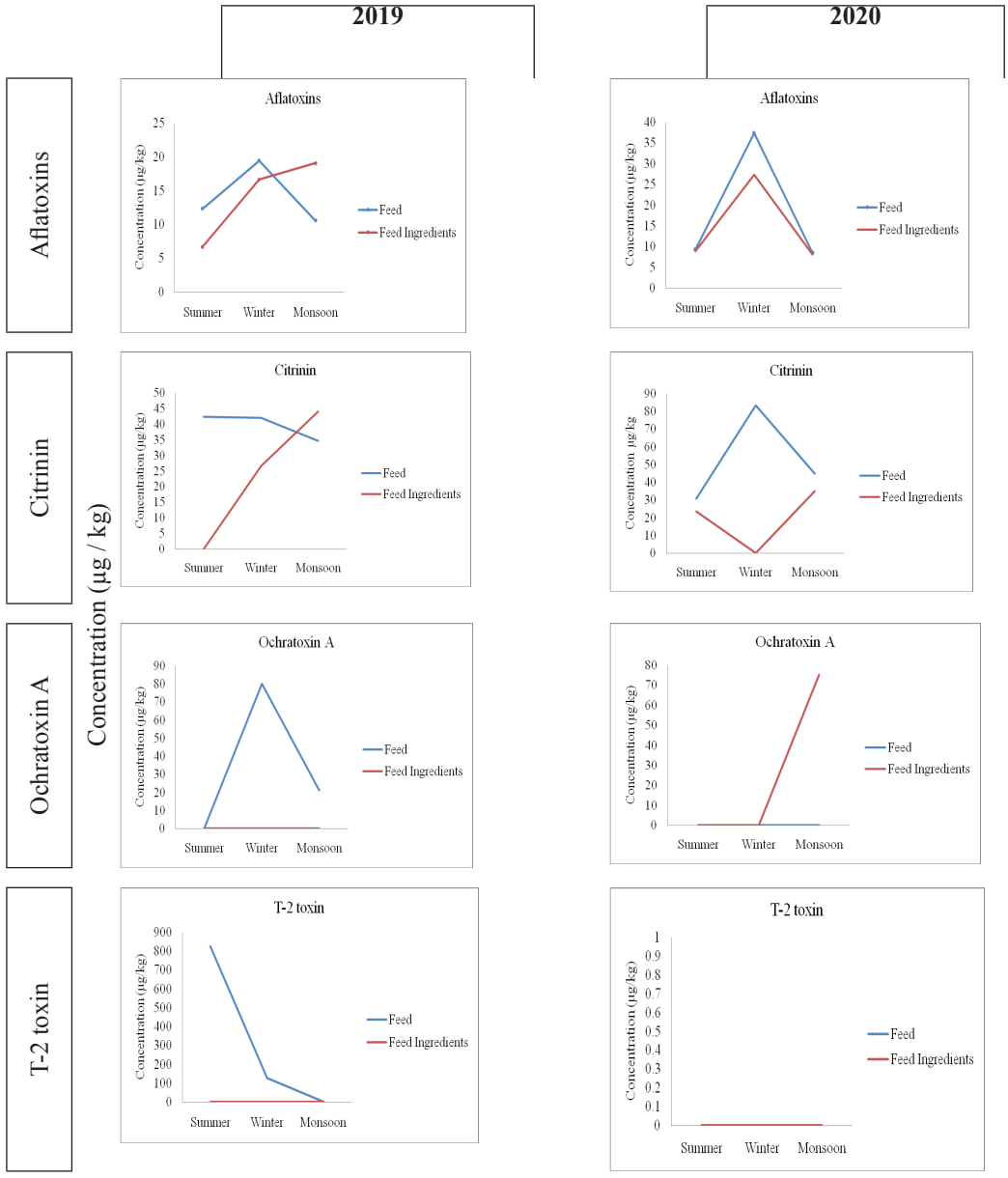


Fig. 3. Season-wise variation in the mycotoxin contamination detected in the analyzed samples for the year 2019 and 2020. The vertical axis shows the mean concentrations of mycotoxins. The horizontal axis shows sample receiving seasons (summer, winter and monsoon) of the years 2019-2020. Blue colour lines indicate feed samples and red colour lines indicate feed ingredients samples.

transport. The present study revealed the occurrence and co-occurrence of mycotoxins in animal feed and feed ingredients received at PLAFFS for the years 201-2020. Aflatoxin B1 was present predominantly in the analyzed feed and feed ingredients either alone or along with multiple mycotoxins. With respect to seasons, there was the highest level of mycotoxin contamination in the winter due to the prevailing conducive climatic conditions for optimal growth of toxigenic fungi and mycotoxins synthesis. In contrast, the concentrations of mycotoxins in many of the analyzed feed samples were above the maximum permissible limit, the consumption of which by the animals may pose a health hazard to livestock and human health. It has been pointed out that the contamination of mycotoxins in feed of food-producing animals can result in residues of the ingested mycotoxins or its metabolites in meat, milk and egg (Pons 1997). The continuous monitoring of the incidences of mycotoxin contamination in feed and feed ingredients is necessary to promote the food animal's health and performance. It is essential to increase the farmer's awareness on the existence of mycotoxins in feed and feedstuff and the method of preventing the same to provide quality feed and feed ingredients to the livestock.

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