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Assessment of total aflatoxin (AFB1, AFB2, AFG1 and AFG2) in fish feed and feedstuffs by using high performance thin layer chromatography

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Abstract
Aflatoxin contamination happens if the storage condition of feed is poor or low-quality ingredients were used for the preparation of feed. The present study aimed at evaluating the level of total aflatoxin contamination in the commercial fish feed and feedstuffs collected from Tamil Nadu, India. Total 70 samples comprising 20 fish feed, 10 corn, 10 sunflower meal, 10 soybeans, 10 wheat bran, 10 groundnut oil cake were analyzed for the presence of total aflatoxin. Romer's all-purpose method was used for extraction and aflatoxin levels were detected by HPTLC. The outcome of this study revealed that the fish feed and feedstuffs contaminated with aflatoxin B1, B2, G1 and G2 were ranged between 10 - 80, 10 - 35, 10 - 20, 10-25 µg kg^{-1} and the percentage of contamination was 88%, 84%, 70%, 54.4%, respectively. Out of 70 samples, 45 samples were contaminated with aflatoxin B1 and the detected levels were above the permissible limit recommended by EU and FDA. The study warrants the need for periodical monitoring of fish feed and feedstuffs to aflatoxin analysis, thereby advocate the need to establish a proper regulatory measure for aflatoxin level in aquaculture feed and feedstuffs to ensure food safety.

Keywords: Aflatoxins, high-performance thin layer chromatography, feed safety, fish feed, feedstuffs

1. Introduction
In the aquaculture industry fish feed is the major cost item and it accounts for 40-50% of the total production cost in the intensive fish farming (Enyidi *et al.*, 2017) [12]. It has been reported that more than 200 fish species and crustaceans are depending on commercial manufacture feed. Fish meal is the major source of lipid and dietary protein content for higher tropical level fishes and crustacean species. Nowadays, the use of fish meal from the aquaculture sector is seem to be decrease. The reason behind the decreased use of fish meal is due to high price and availability of more cost-effective dietary fish meal replacers originated from plant-based ingredients (Davis and Sookying, 2009; Manomaitis, 2009; Tacon *et al.*, 2011) [7, 21, 37]. Plant-derived components are efficiently replacing fish-meal from the finished fish feed. However, using the plant-derived ingredients in feed increases the risk of mycotoxins and fungal contamination and it shows a greater occurrence of mycotoxicosis in fish (Oliveria and Vasconcelos, 2020) [25]. There are several mycotoxins reported, however, the most commonly observed mycotoxins are associated with human health and livestock includes aflatoxins, fumonisin, ochratoxin A, patulin, zearalenone and deoxynivalenol (WHO, 2018) [38]. Aflatoxins are Furanocoumarin derivatives produced by several strains of *Aspergillus parasiticus* and *Aspergillus flavus* through a polyketide pathway and they can contaminate feed and crop and cause significant health complications to animals and humans (Bennett and Klich, 2003) [3]. Aflatoxin contamination is very common in the crop, especially containing high levels of lipids and starch such as maize, soybean, peanut, sunflower, wheat and groundnut oil cake. These are the major ingredients that are used for the formulation of fish feed (Ostrowski-Messner *et al.*, 1995) [26]. Contamination with aflatoxin can also happen if the storage condition of feed is poor or low-quality ingredients were used for the preparation of feed (Schoenthal, 1967) [34]. It is the main problem in aquaculture that leads to health issues and financial losses in the fisheries sector (Shane, 1993; Chavez Sanchez *et al.*, 1994; Santacroce *et al.*, 2008) [36, 6, 33].
Aflatoxin may decrease the productivity of aquaculture, as aflatoxicosis usually leads to decreased body weight, loss of growth and reduce disease resistance capacity and increase mortality levels in fish.

Aflatoxin also bio accumulates in the muscles of the fish and in turn results in aflatoxin residues in fishery products. Fish consumption may become an alternative way for aflatoxin to enter the human food web, posing a risk to the safety of food and human health due to its carcinogenicity, genotoxicity and immunosuppressant effect (El-Sayed and Khalil 2009; Deng *et al.*, 2010; Oliveria and Vasconcelos, 2020) [11, 8, 25]. There are more than 20 aflatoxins are available from which aflatoxin B1, aflatoxin B2, aflatoxin G1, and aflatoxin G2 (AFB1, AFB2, AFG1, AFG2) are four significant aflatoxins (Inan *et al.*, 2007) [17].

Complete elimination of any natural toxicant from feed, feedstuff and food is a difficult objective. Therefore to ensure food and feed safety, the maximum acceptable level is set by regulatory agencies (Bennett and Klich, 2003; EI-Sayed and Khalil, 2009) [11, 3]. The United States Food and Drug Administration (FDA) and the European Union (EU) had already implemented an acceptable level of 20 µg kg^{-1} for aflatoxin in animal feed and ingredients. The Indian regulation, Food Safety and Standard Authority of India (FSSAI) has set the maximum permissible limit for aflatoxin, which is 15 µg kg-1 for cereals and oilseeds (FSSAI, 2016) [14]. In order to avoid the toxicity of aflatoxins, the maximum residual limit should be considered in feed and feedstuffs. Analytical identification and quantification of aflatoxins contaminant even at low level must be carried out with a reliable method. It is necessary to provide fast, accurate and reproducible results to allow a successful control over possible contaminant. Screening and detection of aflatoxins in fish feed and feedstuffs are done by using High-Performance Thin Layer Chromatography.

Although many works have been undertaken on aflatoxins contamination in fish feed and ingredients all over the globe, very few studies in India have been reported. Moreover, in Tamil Nadu, no studies have been undertaken on the analysis of aflatoxins in fish feed and feedstuffs. The purpose of this study is to analyze the level of aflatoxins (AFB1, AFB2, AFG1, AFG2) in fish feed and feedstuffs in Tamil Nadu, India.

2. Material and Methods

2.1 Fish feed and feed ingredients samples collection

In the present study, samples were collected from Thanjavur and Thiruvarur districts of Tamil Nadu, India. The average temperature and relative humidity at the time of sample collection were 36 °C and 81 percent respectively. A total of 70 samples were collected, from which 50 samples feedstuffs and 20 samples were fish feed. The details of the number of samples collected are given in Table 1.

In Tamil Nadu, fish farmers mostly culture tilapia, GIFT tilapia, catfish, Indian major carps and exotic carps which were fed with commercially manufactured fish feed. The information was collected with the help of regional Fisheries Officers. The fish feed was collected from Thanjavur and Thiruvarur based on the differences in size and protein content. The feedstuffs were collected from several feed mills, fish farms and local feed ingredient shops. Every 20 kilograms bag was divided linearly according to its length (upper layer, central layer and lower layer) from which samples (1 kg each) were collected in cloth bags free from moisture. The samples were appropriately labelled with lab unique ID. Sample collection is done as per the concept of the Romer® guide on "Sampling and sample preparation for mycotoxins analysis" (Richard, 2000) [29].

Table 1: Sample collection from Tamil Nadu, India.

Feed category	Thanjavur	Thiruvarur	Total
Fish Feed	10	10	20
Feed ingredients			
Corn	5	5	10
Soybean	5	5	10
Sunflower meal	5	5	10
Wheat bran	5	5	10
Groundnut oil cake	5	5	10
Total	35	35	70

2.2 Reagent

All of the reagents were of analytical grade (Emerck). The Mycotoxins reference standards aflatoxin B1, B2, G1, G2 were procured from Sigma Aldrich, USA. These standards were calibrated and checked for its purity by UV spectrophotometer. The aflatoxin stock and working standard solution were prepared by dissolving in specific solvents to get the desired stock concentration 10 ng/µl (10 ppm) aflatoxin B1, B2, G1, G2 in benzene: acetonitrile 98:2 in ratio.

2.3 Methodology for aflatoxin extraction

The methodology adopted for extraction and cleanup was widely known as the Romers all-purpose method. Fish feed and feedstuffs were finely grind using an electric mixer. 25 g of sample was taken for extraction. After extraction, the dried extract was eventually re-dissolved in 0.2 ml of chloroform and used to spot for HPTLC (Ramesh *et al.*, 2013) [27]. All the analyses were carried out in triplicate and the results were expressed as an average of three repetitions.

2.4 Estimation of aflatoxin B1, B2, G1, G2 by High-performance Thin Layer Chromatography (HPTLC)

In this experiment, 20×10 cm format of silica gel HPTLC plates were used. For quantitative analyses and reproducibility studies, plates were prewashed. The plate was developed with 20 ml of methanol per trough in a twin-trough chamber (TTC) of 20×10 cm size (E Merck). In the present study, Linomate-5 sampler applicator was used, by utilizing Linomate-5 sample applicator, the dried sampler is implemented as bands (spray-on technique). The mobile phase used for the plate was chloroform: acetone in 9:1 ratio. For analyzing the levels of aflatoxins contamination in samples, CAMAG HPTLC scanner-3 is used under 366nm wavelength. Detection of aflatoxins is carried out by high-performance thin-layer Chromatography based on their fluorescence under UV light, whereas aflatoxins require derivatisation for fluorescence enhancement and thus confirmation in the samples (Ramesh *et al.*, 2013) [27].

3. Results

The obtained results show that due to improper handling and storage practices there was an increased risk of aflatoxins production. In our study, four different aflatoxins were analyzed from the fish feed and feedstuffs. AFB1, AFB2, AFG1 and AFG2 were present in 88%, 84%, 70%, and 51% respectively. In 45 samples the content of AFB1 exceeded the FDA and EU limit.

The analysis of aflatoxins shows that Aflatoxin B1 and Aflatoxin B2 contaminated most of the fish feed and feed ingredients. Out of 50 feed ingredients samples analyzed, AFB1, AFB2, AFG1 and AFG2 was detected in 44, 39, 31 and 29 samples respectively. Similarly, out of 20 fish feed

samples analyzed, AFB1, AFB2, AFG1 and AFG2 was detected in 18, 20, 18 and 8 samples respectively. The ranges of the above-mentioned aflatoxins in fish feed and feed ingredients are given in Table 2 and 3.

Table 2: Aflatoxin content (µg /Kg^{-1}) in fish feed.

Mycotoxin	No. of sample Tested	No. of sample contaminated	% of Contamination	Range (µg Kg^{-1}) (Min-Max)	Mean ± S.E. (µg Kg^{-1})
AFB_1	20	18	90%	20-35	22.05 ±1.84
AFB_2	20	20	100%	10-14	10.8 ±0.29
AFG_1	20	18	90%	10-12	9.1 ±0.70
AFG_2	20	8	40%	(10)	4 ±1.12

The above data are mean values of triplicate determinations

As indicated in Table 4, our data shows that samples analyzed from Thiruvarur were the most contaminated with AFB1, with a mean concentration of 27±3.4 µg kg^{-1} followed by Thanjavur 26.8±3 µg kg^{-1}. Aflatoxin B2 shows the higher concentration in Thanjavur (12±1.4 µg kg^{-1}) followed by Thiruvarur (8.8±0.8 µg kg^{-1}). AFG1 and AFG2 show higher concentration in Thanjavur with a mean concentration of 8.8±1 µg kg^{-1}, 6.7±1.2 µg kg^{-1} respectively. Followed by Thiruvarur 6.5±0.8 µg kg^{-1}, 5.4±0.8 µg kg^{-1}respectively.

As indicated in Table 3, Groundnut oil cake was the most contaminated feed ingredient with a maximum average AFB1 range is 80 µg kg^{-1}, AFB2 range is 35 µg kg^{-1}, AFG1 range is 25 µg kg^{-1} and AFG2 range is 25 µg kg^{-1}, followed by wheat bran maximum AFB1 range is 40 µg kg^{-1}, AFB2 range is 15 µg kg^{-1}, AFG1 range is 15 µg kg^{-1}, AFG2 range is 10 µg kg^{-1}, corn maximum AFB1 range is 35 µg kg^{-1} AFB2, AFG1 and AFG2 have 10 µg kg^{-1}, Soybean maximum AFB1 range is 25 µg kg^{-1}, AFB2 range is 19 µg kg^{-1}, both AFG1 and AFG2 have 10µg kg^{-1}, sunflower meal maximum AFB1 range is 25 µg kg^{-1} and AFB2, AFG1, AFG2 have 10 µg kg^{-1}.

The RF value observed for different aflatoxins by HPTLC were 0.55 (aflatoxin B1), 0.51 (aflatoxin B2), 0.43 (aflatoxin G1), 0.40 (aflatoxin B2) and the chromatogram for total aflatoxin standards is shown in figure 1. The matrix-based calibration curves for aflatoxin were prepared by plotting integrated areas on Y axis versus concentrations on the X-axis and the Peak area Vs Concentration is depicted in figure 2.

4. Discussion

4.1 Aflatoxin level in fish feed and feed ingredients

Aflatoxin contamination associated with feed and feedstuffs is a global problem, mostly in the tropical and subtropical regions of the world. It is a common problem in aquaculture that poses both economic and health concern in fishery production, especially in developing countries. The present study confirms the occurrences of aflatoxin contamination in fish feed and feedstuffs from Tamil Nadu, India.

In our analysis, 70% of the fish feed samples were found to be contaminated within a range of 20 to 35 µg kg^{-1} which is almost similar to Mwihia, *et al.* (2018) [24] who have recorded that 84% of the fish feed samples had been contaminated with AFB1 in the range of 1.8 to 39.7 µg kg^{-1}. Other studies from temperate countries like Brazil, Iran and Egypt showed that fish feeds were tested positive for aflatoxins in a range of 67.35 µg kg^{-1}, 68.5 µg kg^{-1} and 150 µg kg^{-1} respectively (Hassan, *et al.*, 2011; Barbosa, *et al.*, 2013; Fallah, *et al.*, 2014) [16, 2, 13]. Kholife *et al.* (2019) [18] recorded that almost 43% of fish feed

Table 3: Aflatoxin level (µg Kg^{-1}) analyzed in fish ingredients

Feed Ingredient	N	Aflatoxin B1				Aflatoxin B2				Aflatoxin G1				Aflatoxin G2			
		N*	% of Contamination	Range (µg Kg^{-1})	Mean ±S.E. (µg Kg^{-1})	N*	% of Contamination	Range (µg Kg^{-1})	Mean ±S.E. (µg Kg^{-1})	N*	% of C	Range (µg Kg^{-1})	Mean ±S.E. (µg Kg^{-1})	N*	% of C	Range (µg Kg^{-1})	Mean ±S.E. (µg Kg^{-1})
Corn	10	10	100%	20-35	27.3±1.5	8	80%	10	8.0±1.3	8	80%	10	8.0±1.33	7	70%	10	6.0±1.6
Soybean	10	6	60%	20-25	13.9±3.8	6	60%	10-19	6.9±2.0	4	40%	10	4.0±1.6	4	40%	10	4.0±1.6
Sunflower meal	10	10	100%	10-25	18.5±1.5	7	70%	10	7.0±1.52	8	80%	10	8.0±.1.3	8	80%	10	8.0±1.3
Groundnut Oil cake	10	10	100%	45-80	67.5±3.5	10	100%	25-35	29.0±1.2	3	30%	20-25	6.5±3.3	3	30%	20-25	7.0±3.5
Wheat bran	10	8	80%	10-40	21.0±4.0	8	80%	10-15	10.5±1.8	8	80%	10-15	9.2±1.6	7	70%	10	7.0±1.5

N = total number of the sample analyzed, n*= positive sample, the above data are mean values of triplicate determinations

Table 4: Aflatoxin levels (µg Kg^{-1}) in analyzed fish fed and feed ingredients from two districts of Tamil Nadu, India

Districts	Sample	Aflatoxin B1		Aflatoxin B2		Aflatoxin G1		Aflatoxin G2	
		Mean±S.E. (µg Kg^{-1})	Range (µg Kg^{-1})	Mean±S.E. (µg Kg^{-1})	Range (µg Kg^{-1})	Mean±S.E. (µg Kg^{-1})	Range (µg Kg^{-1})	Mean±S.E. (µg Kg^{-1})	Range (µg Kg^{-1})
Thanjavur	Fish feed	21.0±2.4	20-25	11.2±0.5	10-14	9.0±1.0	10	2.0±1.3	10
	Corn	27.6±2.5	20-35	8.0±2.0	10	8.0±2.0	10	6.0±2.4	10
	soybean	9.0±5.56	20-25	4.0±2.4	10	4.0±2.4	10	4.0±2.4	10
	Sunflower meal	22.0±1.2	20-25	8.0±2.0	10	8.0±2.0	10	8.0±2.0	10
	Wheat bran	23.0±4.0	10-25	12.4±0.6	10-14	11.0±1.0	10-15	8.0±2.0	10
	Groundnut oil cake	64.0±6.4	55-80	30.0±1.5	25-35	13.0±5.3	20-25	14.0±5.7	20-25
Thiruvarur	Fish Feed	2.1±2.8	20-25	10.5±0.34	10-13	9.2±1.0	10-12	6.0±1.6	10
	Corn	27.0±2.0	25-35	8.0±2.0	10	8.0±2.0	10	6.0±2.4	10
	soybean	8.0±4.7	22-25	9.8±3.0	10-19	4.0±2.0	10	4.0±2.0	10
	Sunflower meal	15.0±1.5	10-20	6.0±2.4	10	8.0±2.0	10	8.0±2.0	10
	Groundnut oil cake	71.0±2.9	65-80	28.0±2.0	25-35	N.D	N.D	N.D	N.D
Total Aflatoxin		27.3±2.29	10-80	11.8±1.0	10-35	7.0±0.6	10-25	7.0±0.6	10-25

The above data are the mean values of triplicate determinations

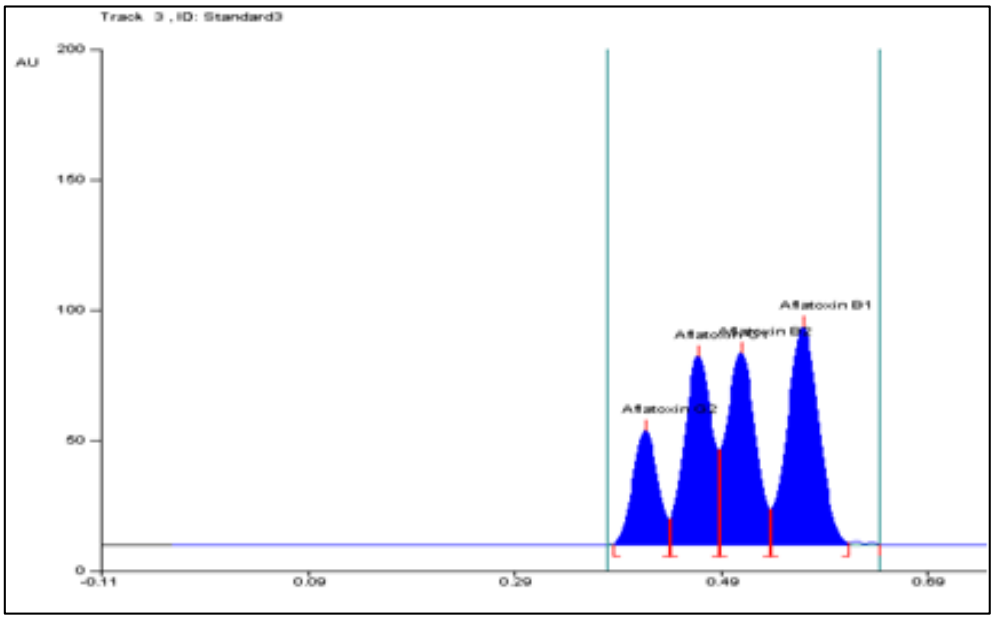
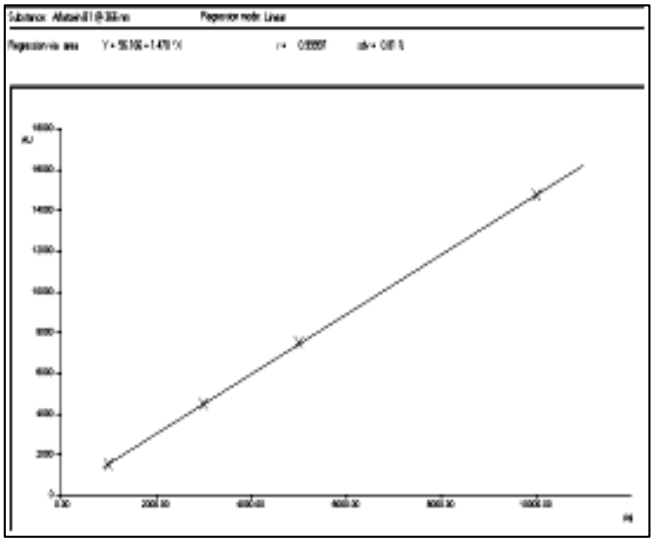
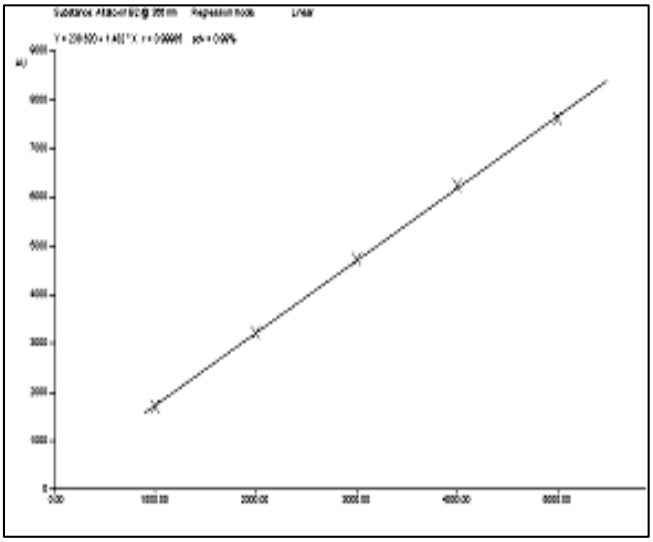


Fig 1: High Performance Thin Layer Chromatography (HPTLC) chromatogram of total aflatoxin



R=0.9999, Aflatoxin B1



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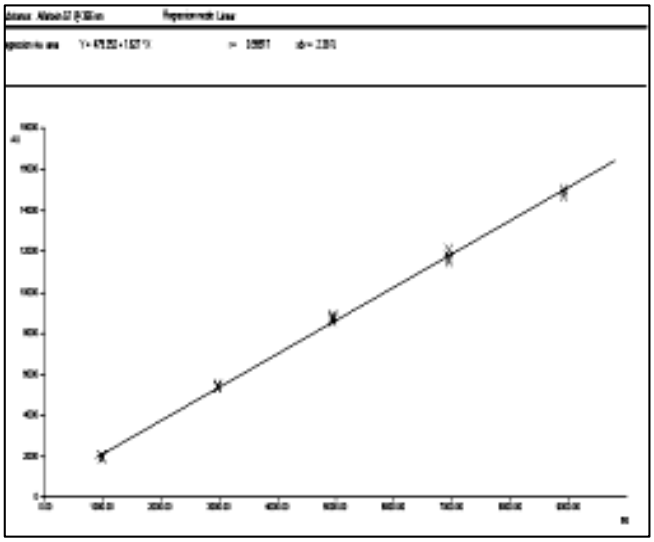
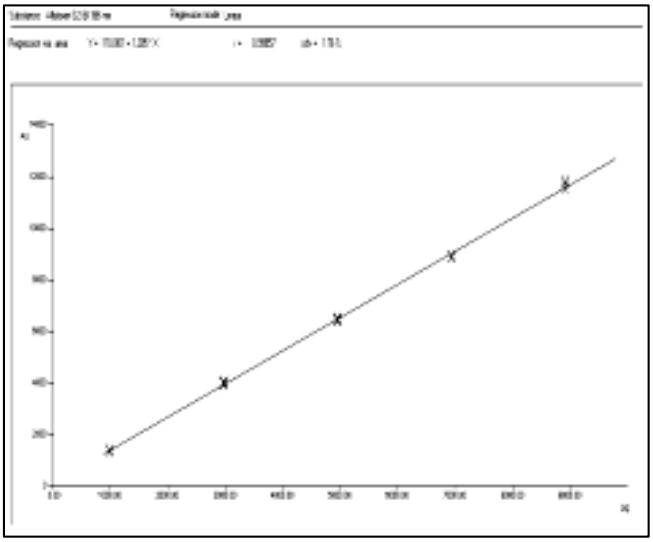


R=0.9991, Aflatoxin G1



R=0.9995, Aflatoxin G2

Fig 2: High Performance Thin Layer Chromatography (HPTLC) matrix based calibration curve of Aflatoxin B1, Aflatoxin B2, Aflatoxin G1 and Aflatoxin

Was contaminated with aflatoxin above the permissible limit of 20 µg kg^{-1}. Marijani *et al.* (2017) [22] reported a higher range of aflatoxins in fish feed in East Africa than the present study i.e. AFB1, AFB2, AFG1 and AFG2 in the maximum level of 806.9, 74.40, 265.60, 65.60 µg kg^{-1}, respectively. Marijani *et al.* (2017) [22] reported an average temperature of 32 °C and relative humidity of 78% during sample collection those were similar to that recorded in the present study 38 °C temperature and 81% humidity . Aflatoxin contamination may happen if the relative humidity is in the range of 70 to 90% and a temperature range of 36 °C to 38 °C (Laly *et al.*, 2019) [20].

Rodrigues *et al.* (2011) [30] and Fallah *et al.* (2014) [13] analyzed aflatoxins contamination in the fish feeds from Brazil and Iran in the maximum level of 67.35 µg kg^{-1} and 68.5 µg kg^{-1} respectively. Dutta and Das (2001) [10] reported 76.2% of aflatoxin contamination in feed. Dutta and Das (2001) [10] and Marijani *et al.* (2017) [22] observed the higher aflatoxin levels due to the higher temperature and high relative humidity, along with improper handling and storage practices of feed.

Reddy *et al.* (2011) [28] and Akinmusire *et al.* (2019) [1] have observed aflatoxin contamination in poultry feeds and ingredients from India and Nigeria. Reddy *et al.* (2002) [28] detected aflatoxin in corn, sunflower meal and groundnut oil cake. Corn and groundnut oil cake contaminated with a higher level of aflatoxin (3300 µg kg^{-1}) and soybean were free from aflatoxin. Akinmusire *et al.* (2019) [1] have detected total aflatoxins (AFB1, AFB2, AFG1, AFG2) in all the feedstuffs samples. AFB1 was detected in 83% of the samples in a range of 0.5 to 760 µg kg^{-1} which was higher than the present study.

Oilseed cakes and cereals are commonly used as essential ingredients for formulating feeds and are mostly subject to contamination with mycotoxins (Bryden, 2012) [4] due to factors such as high energy and protein content, improper handling and climate change. Corn is the primary feed ingredient with high protein and provides adequate nutrition to animals. Dunham *et al.* (2017) [9] reported aflatoxin contamination in corn at a range of 0.0 to 19.91 µg kg^{-1} in Texas. Rodrigues and Naehrer (2012) [31] conducted a study on the prevalence of mycotoxins worldwide, analyzed 4627 samples of soybean, corn, wheat and finished feed. From that 33% of the sample is tested positive for aflatoxin and the highest level of aflatoxin was found in corn samples with a higher range of 6105 µg kg^{-1} from Vietnam. The present study reported 88% of aflatoxin contamination, in which all corn samples were tested positive for aflatoxin in the higher level of 35 µg kg^{-1} which exceeds the FDA limit set for aflatoxin.

Marijani *et al.* (2017) [22] and Mmongoyo *et al.* (2017) [23] analyzed aflatoxin contamination in sunflower meal in the range of 806.9 µg kg^{-1} and 662.7 µg kg^{-1} respectively, which was higher than the present study. Numerous studies have revealed the occurrence of aflatoxins in fish feeds and feedstuffs which are above the permissible limit including the findings of the present study that 45 analyzed samples were above the permissible limit.

4.2 High-performance thin layer chromatography (HPTLC) analysis

Feed and feed ingredients contaminated with the aflatoxin is a global problem, mostly in the tropical and subtropical regions across the globe. It is a common problem in aquaculture that poses both economic and health concerns in fishery production, especially in developing countries. The present study confirms the occurrences of Aflatoxins contamination in fish feed and feedstuffs from Tamil Nadu, India.

Scussel (2003) [35] reported that the analysis of aflatoxin by HPTLC is a cost-effective method as a huge number of samples can be analyzed and quantified per HPTLC plate run. It gives high-quality toxin separation to improve the reproducibility, repeatability, quantification and accuracy of the scanner. Our findings also agreed with the study of Kotinagu *et al.* (2015) [19] and Ramesh *et al.* (2013) [27] who reported that HPTLC is fast with good recovery in the quantitative determination of aflatoxins from feed and ingredients.

4.3 Aflatoxin residues in fish and hazard to public health

The bioaccumulation of aflatoxins from feed to animal food products may represent a serious hazard to public health (CAST, 2003) [5]. There are several studies reported the bioaccumulation of aflatoxin in fish tissue in both tropical and cold water species. Most of the study detected aflatoxin residues in muscle and liver. El-Sayed and Khalil (2009) [11] studied that feeding aflatoxin contaminated feed to fish for a long time can cause health issues in treated fish and also pose a serious risk to the fish consumer through bioaccumulation of aflatoxins in fish musculature. In aquaculture species, transfer factors are quite higher as compared to transfer factors for eggs, whole milk, fat of livestock provenience (Goncalves *et al.*, 2020) [15]. Currently, there are no guidelines or regulation exists to avoid the deposition of aflatoxin in farmed fish or shrimp, and there is not any specific limit set for aflatoxin levels in fish feed. Regulatory limits and guidance values were established based on the reports of terrestrially farmed animals, but in case of aquatic organisms, there is a need to establish a proper regulatory limit for aflatoxin level in aquaculture feed and food to ensure food safety.

Numerous studies have revealed the occurrence of aflatoxins in fish feed and ingredients together with previous evidence, our findings clearly show that aflatoxins are present in fish feed and feed ingredients all over the world.

The present study contributes towards the awareness of aflatoxins contamination in fish feed and feedstuffs, and the use of aflatoxin contaminated feed may cause economic losses and serious health problems in humans due to higher mortality rates, decreases in productivity in fish and aflatoxin residual effects on humans.

5. Conclusion

The present study indicated that the fish feed and feedstuffs are mostly contaminated with aflatoxins. Ingredients particularly cereals and oilseed are the major sources for aflatoxins in fish feed, so it is essential to use good quality ingredients for the formulation of fish feed. Consumption of aflatoxin contaminated feeds may affect the health of fish and can also lead to economic losses to the fish farmers.

Since aflatoxins are produced during storage conditions, it is important to routinely monitor fish feed as well as raw materials. Aflatoxins are often produced in trace concentration, so the sensitivity of the detection system is also essential, therefore it is necessary to introduce mycotoxins detection procedure in the quality control plan of agriculture raw material and finished products. HPTLC is a cost-effective method, it is fast, has good recovery, linearity, reproducible, sensible, precision and accurate in the quantitative determination of aflatoxins from feed and ingredients. It is

necessary to set a proper regulatory limit for aflatoxins level in aquaculture feed and food to ensure food safety.

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