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Assessment of Fungal Contamination in Poultry Feed and Evaluation of Antifungal Potential of Pomegranate Peel Extracts for Feed Preservation

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Abstract

The present study investigated feed samples collected from poultry and cattle farms for moisture, protein, and fat content. Fungal isolates were also enumerated, with Aspergillus flavus being the most predominant. Toxin production potential and presence of aflatoxin genes in A. flavus were evaluated using PCR. Additionally, the antifungal activity of pomegranate peel extracts was examined. Moisture content ranged from 8.95% to 10.47%, while protein content varied between17.14% and 25.11%. Fat content ranged from 0.84% to 2.41%. A. flavus dominated the fungal population, raising concerns about aflatoxin contamination. Phytochemical analysis of pomegranate peel extracts revealed various bioactive compounds. The extracts exhibited substantial antifungal activity against A. flavus. These findings highlight the importance of effective management strategies to control fungal contamination in animal feed and suggest pomegranate peel extracts as potential natural preservatives.

Key Words: Animal feed, Fungal contamination, Aspergillus flavus,aflatoxin, antifungal activity

Introduction

Aflatoxins, potent mycotoxins produced by fungi of the genus Aspergillus, pose a significant threat to both human and animal health. Among these, Aspergillus flavus stands out as a primary culprit, frequently contaminating various agricultural commodities, including poultry feed (Chang et al.,2019). The presence of aflatoxin in feed not only compromises the nutritional value of the feed butalsoleads to the accumulation of toxic residues in animal products, such as meat and eggs, which ultimately find their way into the human food chain (Dong et al., 2017, Bacanlı et al..,2024).

Poultry farming, a crucial component of the global food industry, relies heavily on the quality of the feed provided to the birds. The potential contamination of poultry feed with aflatoxins raises concerns not only about animal health and welfare but also about food safety and security. Variousstrategies have been explored to mitigate aflatoxin contamination, including the use of chemical agents. However, these approaches often come with environmental and health risks (Hassan et al.,2020).

In recent years, there has been a growing interest in exploring natural alternatives for controlling aflatoxin contamination. Plant extracts, rich in bioactive compounds, have emerged as promising candidates due to their potential to inhibit fungal growth and toxin production (Ribeiro et al., 2018). For instance, pomegranate peel, known for its antioxidant and antimicrobial properties, has shown inhibitory effects against various pathogenic microorganisms (Jayaprakasha et al., 2011). Such naturalsolutions not only address the concerns associated with chemical interventions but also align with the increasing consumer demand for safer and more sustainable food production practices.

This study aims to investigate the presence of aflatoxin-producing A. flavus isolates in poultry feed samples and assess the inhibitory potential of plant extracts, particularly from pomegranate peel, against these toxin-producing isolates. The research objectives encompass the collection andanalysis offeed materials, isolation and identification of fungal isolates, molecular identification of aflatoxin- producing isolates, and the preparation and evaluation of plant extracts for their antimicrobial properties.

By elucidating the efficacy of plant extracts in inhibiting aflatoxin-producing fungal isolates in poultry feed, this study contributes to the growing body of knowledge on sustainable and safer approaches for ensuring food security and animal health.

Materials and Methods

Sample Collection

A total of 100 samples of feed mixtures designed for poultry and cattle feeding were collected from various farming areas in Namakkal, Tamil Nadu. The collected poultry feed samples includedthose from chicken broilers and chicken layer farms.

Determination of Moisture Estimation (Alam et al., 2001)

Moisture content was determined using a moisture dish of appropriate size. Approximately 10 g of each sample was weighed and placed in the dish, followed by drying in a hot air oven at 120°Cfor about 2 hours. The dish was then cooled in a desiccator and reweighed to calculate the percentage of moisture.

Determination of Protein

Protein content was determined using the Kjeldahl method as described by Chang (2003). The sample (0.5 g) was digested with concentrated H_2SO_4 and a selenium catalyst. The resulting digest was then subjected to distillation, and the nitrogen content was determined by titration. Protein content was calculated using the formula: Protein (%) = N_2 (%) × 6.25.

Determination of Crude Fat

Crude fat content was determined using a solvent extraction gravimetric method described by Kirkand Sawyer (1980). The sample (5 g) was extracted with petroleum ether in a Soxhlet apparatus, and the extracted fat was dried, weighed, and calculated as a percentage of the sample weight.

Isolation of Fungal Isolates from Feed Samples

Fungal isolates were obtained from feed samples using Sabouraud's Dextrose Agar (SDA Hi- media). Diluted feed samples were spread onto SDA plates and incubated. Colonies were counted,and colony-forming units (CFU) per gram of feed were calculated.

Screening of Toxin-Producing Isolates

Toxin-producing isolates were screened using a coconut-based medium test (Saito et al., 1999). Colonies were inoculated on coconut-based medium plates and observed for the presence of a fluorescence ring under UV light.

Fungal Genomic DNA Extraction

Genomic DNA was extracted from fungal cultures using a lysis buffer containing glass beads. The resulting homogenate was treated with RNase A and subjected to phenol-chloroform extraction. The DNA pellet obtained was dissolved in TE buffer.

PCR Analysis

PCR analysis was performed using primer pairs (Table 1) designed based on A. flavus aflatoxin biosynthetic genes (aflR, aflS, and aflD). Genomic DNA was amplified, and PCR products were analyzed by agarose gel electrophoresis.

Collection of Plant Samples

Pomegranate peels were collected from local markets in Namakkal, Tamil Nadu. The peels were cleaned, dried, and ground to a fine powder.

Extract Preparation

Ground pomegranate peels were soaked in methanol for 24 hours, and the extracts were concentrated using a rotary evaporator. The dried extracts were stored for further use.

Phytochemical Screening of Pomegranate Peel Extract

Phytochemicals in the pomegranate peel extract were screened using various tests for alkaloids, carbohydrates, flavonoids, phenols, saponins, tannins, terpenoids, quinones, and steroids.

Antifungal Activity of Pomegranate Extract

The antifungal activity of pomegranate extract was determined using the agar well method. Wellswere made in agar plates inoculated with fungal isolates, and different concentrations of plant extract were added to the wells. The inhibition zones were measured after incubation.

Results

Determination of Moisture Estimation

Moisture content for all samples was shown in Table 1 and Graph 1. In all feed samples, the moisture content ranged from 8.95±0.25 to 10.47±0.15%. Among the samples, Feed Sample 10 had the highest moisture content, followed by Feed Sample 3, and then Feed Sample 6. The lowest moisture content was observed in Feed Sample 5.

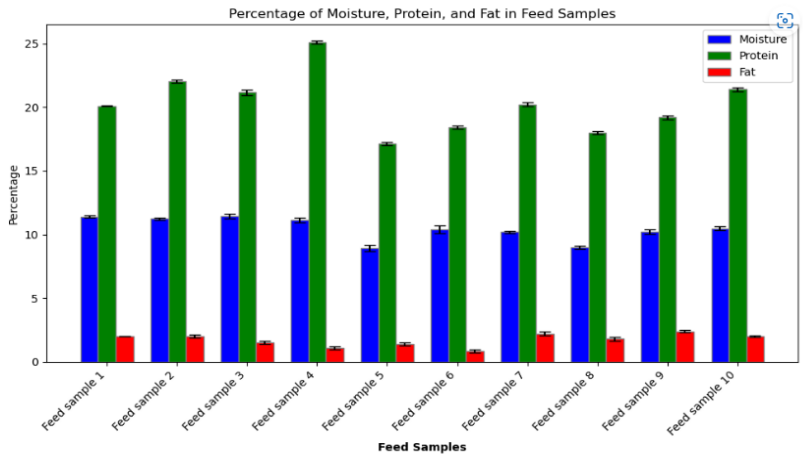
Determination of Protein in Feed Samples

The crude protein content analysis was conducted on all feed samples, and the protein percentage ranged from 17.14±0.11 to 25.11±0.11. Among the ten samples, the highest protein percentage was observed in Feed Sample 4, followed by Feed Sample 2. The lowest protein percentage was observed in Feed Sample 5.

Table 1: Determination of Moisture, Protein and Fat level of feed sample

Names of the samples	% of Moisture	% of Protein	% of Fat
Feed sample 1	11.41±0.11	20.1±0.04	2.0±0.01
Feed sample 2	11.25±0.10	22.01±0.12	2.01±0.11
Feed sample 3	11.45±0.20	21.15±0.21	1.51±0.13
Feed sample 4	11.14±0.18	25.11±0.11	1.11±0.14
Feed sample 5	8.95±0.25	17.14±0.11	1.41 ±0.11
Feed sample 6	10.41±0.32	18.42±0.14	0.84±0.12
Feed sample 7	10.20±0.11	20.22±0.14	2.22±0.14
Feed sample 8	9.0±0.12	18.01±0.11	1.81±0.13
Feed sample 9	10.21±0.17	19.21±0.16	2.41±0.10
Feed sample 10	10.47±0.15	21.40±0.14	2.0±0.07

Graph 1: Determination of Moisture, Protein and Fat level of feed sample



Determination of Fat Level in Feed Samples

Fat content analysis was performed on all feed samples, and the fat percentage ranged from 0.84±0.12 to 2.41±0.10. Among the ten samples, the highest fat percentage was observed in Feed Sample 9, followed by Feed Sample 7. The lowest fat percentage was observed in Feed Sample 6.

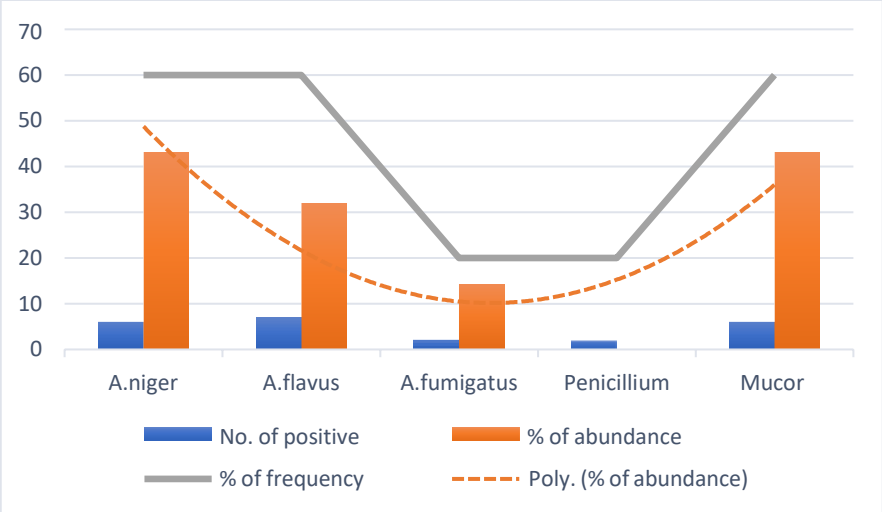
Enumeration of Fungi from Feed Samples

A total of 10 samples were analyzed for fungal isolate enumeration. The highest abundance and frequency were observed for A. flavus, followed by A. niger and Mucor (43%). Among the 10 samples, the highest number of fungal species were observed in Feed Sample 3 (Graph 2).

Table 2: Incidence of fungi isolates on feed samples

Name o the funga isolates	Feed samples										No. o positive	% o abundance	% o frequency
	F1	F2	F3	F4	F5	F6	F7	F8	F9	F10			
A.niger	+	+	-	+	-	-	-	+	+	+	6	43	60
A.flavus	-	+	+	-	-	+	+	+	-	+	7	32	60
A.fumigatus	-	-	+	-	+	-	-	-	-	-	2	14.2	20
Penicillium	-	-	+	-	-	-	-	-	+	-	2	0	20
Mucor	-	+	+	+	+	+	+	-	-	-	6	43	60

Graph 2: Incidence of fungi isolates on feed samples



Isolation of Toxin-Producing Fungal Isolates (A. flavus)

Among the 7 isolates of A. flavus, 4 were positive for toxin production as indicated by the formation of a fluorescence zone under UV light.

Table 3: Isolation of toxin producing isolates of *A.flavus*

S.No	Names of the samples	Types of aflatoxin genes			Fluorescence test: Toxin
		aflR	*aflS*	*aflD*	
1	*A. flavus1*	+	-	+	+
2	*A. flavus2*	-	-	-	-
3	*A. flavus3*	-	-	+	+
4	*A. flavus4*	-	-	-	-
5	*A. flavus5*	-	-	-	-
6	*A. flavus6*	+	-	+	+
7	*A. flavus7*	+	+	-	+

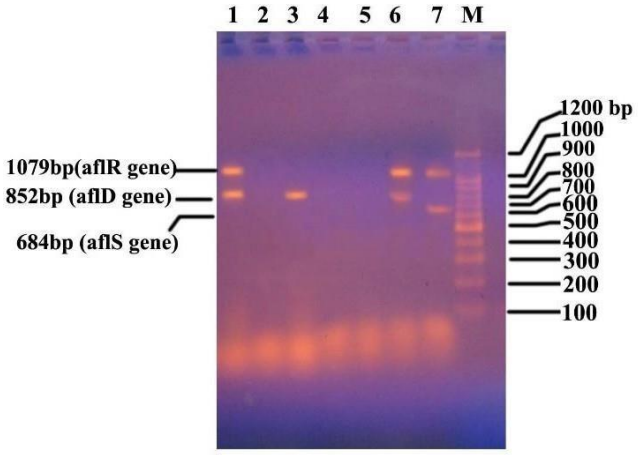
Amplification of Aflatoxin-Producing Genes by PCR

PCR amplification was conducted on 4 isolates for aflatoxin-producing genes. The predominant genes were *aflD* and *aflR*, followed by *aflS* (Table 1). All isolates had at least a single gene, exceptisolates 4 and 2. Four different types of gene patterns were observed among the 7 isolates. The results are tabulated in Table 4.

Table 4. Aflatoxin primer sequences

Gene	Sequence	PCR product size (bp)
aflR	**5'-AAGCTCCGGGATAGCTGTA-3'** **5'-AGGCCACTAAACCCGAGTA-3'**	**1079**
aflS	**5'-TGAATCCGTACCCTTTGAGG-3'** **5'-GGAATGGGATGGAGATGAGA-3'**	**684**
aflD	**5'-CACTTAGCCATCACGGTCA-3** **5'-GAGTTGAGATCCATCCGTG-3'**	**852**

Figure 1: Amplification of aflatoxin A.flavus



Lane:1-*A. flavus*1, Lane:2-*A. flavus2*, Lane:3-*A. flavus*3, Lane:4-*A. flavus*4
Lane:5-*A. flavus*5, Lane:6-*A. flavus*6, Lane :7-*A. flavus*7

Preliminary Phytochemical Analysis of Pomegranate Peel Extract

Phytochemical analysis of pomegranate peel extract was conducted using methanol and chloroform solvents. Methanol extract showed positive results for alkaloids, carbohydrates, flavonoids, phenols, sterols, terpenoids, and tannins. Chloroform extract did not show the presenceof flavonoids, phenols, saponins, protein, and quinones. Methanol extract exhibited higher percentages of phytochemicals (Table 5)

Table 5: Preliminary phytochemical analysis of pomegranate peel extract

S.No	Phytochemicals	Methanol	Chloroform
1.	Alkaloids	+	+
2.	Carbohydrates	+	+
3.	Flavonoids	+	-
4.	Phenols	+	-
5.	Saponins	-	-
6.	Sterols	+	+
7.	Tannins	+	+
8.	Terpinoids	+	+
9.	Quinines	-	-
10.	Protein	-	-

Antifungal Activity of Methanol Extract against Aflatoxigenic Fungi

Methanol extracts were used for antifungal activity due to higher phytochemical content. Amongthe 4 isolates, 3 were suppressed by peel extract with zone of inhibition ranging from 9mm to 18mm. Different concentrations of pomegranate peel extracts were tested, and the highest resistance was observed at 10mg of extract (Table 6, Figure 2).

Figure 2: Antifungal activity of Pomegranate peel extract againstAflatoxin producing A.flavus

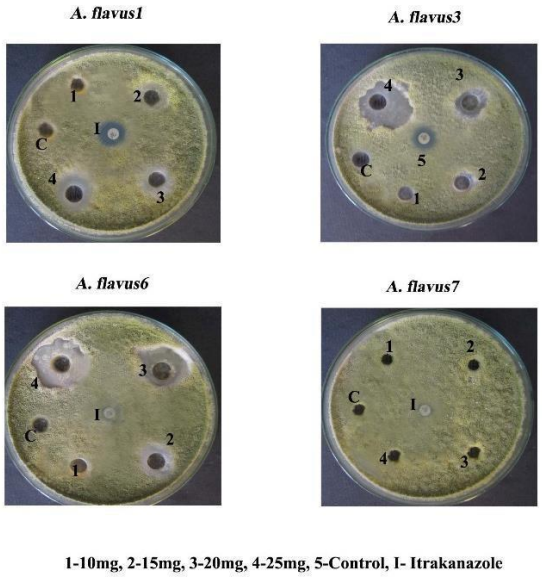
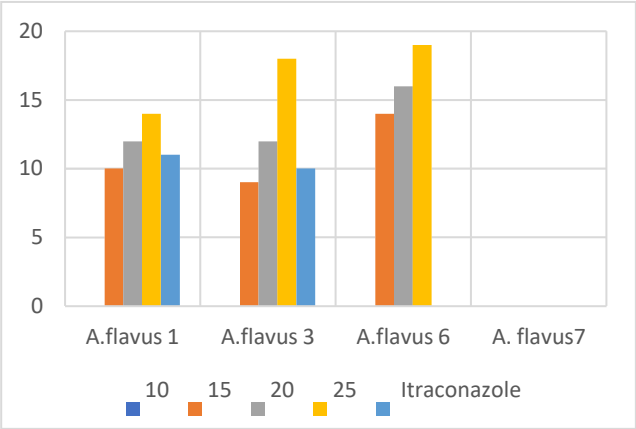


Table 6: Antifungal activity of methanol pomegranate peel extract

S.No	Isolates name	Con. of extracts (mg) Zone of inhibition in mm				
		10	**15**	**20**	**25**	***Itraconazole***
1.	*A.flavus 1*	-	10	12	14	11
2.	*A.flavus 3*	-	9	12	18	10
3.	*A.flavus 6*	-	14	16	19	-
4.	*A. flavus7*	-	-	-	-	-

Graph 3. Antifungal Activity of Methanol Extract against Aflatoxigenic Fungi



Discussion

The present study aimed to determine the aflatoxin-producing *Aspergillus flavus* isolated from poultry feed samples and to inhibit its growth using plant extracts. The obtained results shed lighton various aspects of feed composition, fungal enumeration, toxin production, and potential antifungal activity. Moisture content is a critical factor in determining the stability and quality of feed materials. The moisture levels observed in the current study (8.95-10.47%) fall within the range reported by other researchers (Alam et al., 2001) for poultry feed materials. High moisture content can lead to fungal growth and mycotoxin production, highlighting the need for proper storage and handling.

The protein content of the feed samples varied from 17.14% to 25.11%. These values align with the findings of Chang (2003) and reflect the diverse nutrient profiles of different feed mixtures. Protein content plays a crucial role in animal nutrition and health, and its variation in feed can impact livestock productivity.

The fat content in the samples ranged from 0.84% to 2.41%, showing variations in feed formulations. Similar findings were reported by Kirk and Sawyer (1980), highlighting the importance of fat as an energy source for animals. However, excessive fat levels can lead to rancidity and fungal growth in feed.

Fungal enumeration revealed the prevalence of *A. flavus* and *A. niger*, which are common contaminants in feed materials. The dominance of *A. flavus* is concerning due to its ability to produce aflatoxins, potent mycotoxins that can contaminate feed and pose health risks to animalsand humans. These results are in agreement with studies by Ahmed, et al. (2023) and emphasize the need for effective control measures.

The isolation of toxin-producing isolates (*A. flavus*) indicated that a subset of *A. flavus* isolates possessed the potential to produce aflatoxins. This finding underscores the importance of monitoring and managing fungal contamination in feed materials, as aflatoxins pose serious healthhazards.

The PCR analysis for aflatoxin-producing genes revealed variations in gene presence among different isolates. This suggests genetic diversity within *A. flavus* populations, which could influence toxin production. Similar observations were made by Antonia et al. (2012) and Perez- Corrales et al., (2024), emphasizing the need for molecular characterization to understand toxin- producing potential.

Phytochemical analysis of pomegranate peel extracts indicated the presence of bioactive compounds such as alkaloids, carbohydrates, flavonoids, phenols, sterols, terpenoids, and tannins. These compounds have been reported to exhibit antimicrobial properties (Ugochukwu et al., 2013, Krishnaveni et al., 2023), which could explain the observed antifungal activity against *A. flavus*. The inhibition of fungal growth by pomegranate extracts suggests their potential application as natural preservatives in feed.

The antifungal activity of methanol pomegranate peel extract against *A. flavus* isolates highlightedits effectiveness in inhibiting fungal growth. The observed inhibition zones provide promising evidence of the extract's antifungal potential. These findings resonate with the work of recent studies that have investigated the antimicrobial activity of plant extracts against fungal pathogens (Alam et al., 2013, Ahmed et al., 2023)

In conclusion, this study has provided significant insights into various aspects of poultry feed composition, including fungal contamination, toxin generation, and possible strategies for countering these issues. The outcomes emphasize the importance of implementing efficientmanagement protocols to curtail fungal contamination and toxin synthesis within feed components, consequently upholding the safety of animals and humans alike. The inherent antimicrobial attributes found in pomegranate peel extracts present a hopeful prospect for pioneering the creation of organic substitutes to conventional preservatives. By incorporating pomegranate peel, an agricultural byproduct, as an additional component in poultry feed, there is potential to mitigate toxin levels and further enhance thesafety and quality of the feed supply chain. This innovative approach aligns with sustainable practices in agriculture while also contributing to the overall health and productivity of poultry.

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