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Inhibition of fungal growth and aflatoxins biosynthesis of *Aspergillus flavus* and *Aspergillus parasiticus* using *Azadirachta indica* seed cake residue and its application to decontaminate poultry feed

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Abstract

Contamination of aflatoxins (AFs) produced through *Aspergillus* species postures a stern danger to poultry feed safety and animal health. This study assessed the Fourier-transform infrared spectroscopy (FTIR) analysis, antifungal and anti-aflatoxigenic potential of neem seed cake residue powder (NSC) against *Aspergillus parasiticus* and *Aspergillus flavus* in poultry feed during storage. The NSC powder Fourier-transform infrared spectroscopy (FTIR) analysis displayed eighteen characteristics peaks (3294.42–597.93 cm^{-1}), representing the occurrence of various functional classes i.e. aromatic, amide, carbonyl, C–H, aliphatic and hydroxyl groups, signifying bioactive substances accredited for antifungal activities. Poultry feed was blended with various dosage of NSC (1–20% w/w) and checked for fungal growth, sporulation and AFs synthesis over time of six weeks and six months, respectively. Fungal growth was evaluated applying a visual scoring system, whereas sore counts and AFs quantity (AFG2, AFG1, AFB2 and AFB1) were measured applying standard analytical techniques. The NSC significantly controlled ($P < 0.05$) fungal development and spore synthesis in a dose dependent way. Higher NSC dose (15–20% w/w) efficiently inhibited mycelial growth of both mould species and minimized spore quantity to negligible levels. The accumulation of AFs raised gradually in untreated controls while was significantly minimized in NSC-treated samples. At 20% NSC, AFs (AFB1 and AFB2) level were minimized 87% and 74% against *Aspergillus flavus* respectively, while AFB1, AFB2, AFG1 and AFG2 inhibited 74%, 85%, 75% and 76% against *Aspergillus parasiticus* respectively. Generally, NSC documented powerful ability as eco-friendly, economical and natural feed additive for suppression moulds contamination and minimizing AFs risks in poultry feed during storage.

Keywords: Neem, toxic fungi, spore's inhibition, natural preservatives, toxin inhibition.

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1. Introduction

Aflatoxins are teratogenic, immunosuppressive, mutagenic, carcinogenic and poisonous secondary metabolites synthesized through *Aspergillus nomius*, *Aspergillus parasiticus* and *Aspergillus flavus*. These moulds cultivate universally and pollute agriculture commodities and crops like animal feed, cheese, milk, oil seeds (soybean, sesame, sunflower and cotton), peanuts, Brazil nuts, walnuts, almonds, pistachios, turmeric, coriander, ginger, chili, black paper, sorghum, barley, maize, wheat and rice

(Jaspal *et al*, 2023). Aflatoxins (AFs) are the greatest researched poisons because of its linked with high mortality and morbidity rate in poultry. Therefore, AFs in various feed grains are a major public healthiness alarm, which unfavorably affects animal health and human (Naveed *et al*, 2022). Aflatoxins are resistant to degradation and stable. Among the 18 various categories, AFs (M2), AFs(M2), AFs(G2), AFs(G1), AFs(B2) and AFs(B1) are the chief categories and derivative of bifuranocoumarins. The Food and Agriculture (FAO) predicted that around 25% of the globe's cereals are polluted by mycotoxins, counting AFs (Jaspal *et al*, 2023). Amongst poultry and livestock, these toxins are mostly accountable for aflatoxicosis and the illness leads to severe defeat of the immune system and a minimum production rate (Yang *et al*, 2020). Feed and food contamination with moulds toxins minimize its quality and safety account an enormous damage at the manufacturing level. Though, Food and Drug Administration (FDA) reflects AFs as an inevitable food pollutant and has set regulatory degree for it. The standard level for poultry is 20ppb (Naveed *et al*, 2022). The AFs G2 and G1 produce a yellowish green color, while AFs B2 and B1 give blue color under UV light. The AFs (M) are hydroxylated derivatives of AFs B and first isolated from milk. The Aspergillus parasiticus synthesize AFs (G2, G1, B2 and B1), while Aspergillus flavus synthesize only AFs (B2 and B1), but it is also able to produce cyclopiazonic acid (Jaspal *et al*, 2023). The production of mycotoxins might take place in either pre-harvest stage of crop. In addition to feed, AFs can also be originated in dairy milk products of livestock bare to these feeds. The ecological factors straightly effecting the AFs syntheses comprise moisture contents, oxygen and temperature. Generally, the synthesis of AFs (G) happens at 28°C whereas that of AFs (B) characteristically happens at 11-37°C (Obrian *et al*, 2007). As the temperature in various areas rises because of unexpected global changes, a maximum temperature might ease the fungi growth, such as Aspergillus species that embellishment in warmer weather areas. This, in turn, results in increased AFs synthesis (Naveed *et al*, 2022). Pakistan is one of the countries exaggerated harmfully accredited to climate change. Consequently, an augmented danger of AFs is predictable in the next few years. In Pakistan, the poultry industry donates 26.8% to total meat production. The poultry category has raised as an origin of service for more than 1.5 million persons in the previous few years (Sadiq, 2004). Poultry feed and ingredients have been regularly contaminated through AFs in various areas of Pakistan (Anjum *et al*, 2011; Hanif *et al*, 2005; Rashid *et al*, 2012). Numerous studies have been documented various ratios of AFs (B1 and B2) pollution in the poultry feed starter chick and poultry starter broiler with dosages ranging from 100 and 320µg/kg (Naveed *et al*, 2022). According to the study findings, 92.5% of samples were observed positive for the AFs detection ranging between 34 and 86.2ppb (Naveed *et al*, 2022).

It is broadly acceptable that the application of synthetic pesticides to control post-harvest diseases has been inadequate because of its environmental contamination, lengthy degradation time, teratogenicity, carcinogenicity and their contrary properties on human health and food. The AFs inactivation using chemical and physical technique has not yet showed to be economically feasible and effective (Jaspal *et al*, 2023). Instead of synthetic pesticides, plants extracts have delivered a substitute to protect feed and food from mould pollution and AFs formation. The extracts of plants will most probable to be the best to utilize ad food additives in various food industries to stop deterioration and contamination through AFs producing moulds. Post-harvest treatments to suppressed AFs such as gamma radiation, heat, ammonization and alkalization are not commonly used by growers (Jaspal *et al*, 2023).

The neem (*Azadirachta indica*) is applied as a domestic pesticide. The oil or extract of neem is active against immuno modulatory, antipyretic, analgesic, inflammatory, antiulcer, antidiabetic and immune-stimulant activities (Ali *et al*, 2022). The antifungal agents' rich content in plants are being utilized as biopesticide since up to the commencement of human civilization. Antifungal properties of plants and its products arise clearly every day. Antifungal compounds which are gained from herbs have biodegradable and less side effect against environment therefore providing a noteworthy benefit. Currently, a marketable pesticide applied against crop disease is found to cause injury to human health and ecosystem. Because of that leading a research of substitute management techniques comes into distinction for reduction applied marketable pesticides. Research originate that substances in the shape of herbs and essential oils were observed antiviral, herbicidal, nematicidal, nematicidal, insecticidal, antibacterial and antifungal properties.

Regardless of widespread research on neem and neem-based products, limited studies have discovered the combined application of *Azadirachta indica* seed cake for Fourier Transform Infrared (FTIR) Spectroscopy based characterization, antifungal activities (spores' inhibition and mould growth) and aflatoxins biosynthesis inhibition in poultry feed. So, the current study goals to develop a sustainable and multifunctional approach applying neem seed cake residue for mould control, inhibition of toxins and poultry feed decontamination. This study provides innovative visions into the association between functional groups (FTIR) and antifungal and anti-aflatoxigenic activities, as well as a cost-effective approach for poultry feed protection. The current study was carried out to determine the neem seed cake residue FTIR profiling, antifungal activities and its application in poultry feed to suppress the aflatoxins production for six-month storage.

2. Materials and Methods

2.1. Collection of Neem Product

The Neem Seed Cake Residue Powder (NSC) were obtained from Herbal Pharmaceutical Pilot Plant of Medicinal Botanic Centre (MBC) of Pakistan council of Scientific and Industrial Research (PCSIR), Peshawar- Pakistan.

2.2 FTIR analysis of NSC

The FTIR method was applied for the detection of diverse functional category in NSC. The infrared spectroscopy (IR) spectrum was attained applying FTIR Prestige -21 Shimadzu Japan. The sample was scanned from 3,900 to 600 cm^{-1}, operating at a resolution of 4 cm^{-1}, with 10 number of scans.

2.3. Fungal Cultures

The pure fungal strains of *Aspergillus flavus* (Accession No 1000) and *Aspergillus parasiticus* (Accession No 1002) were collected from Mycotoxin Research Section of Food Microbiology Center of PCSIR Laboratories Complex Jamrud Road Peshawar, Khyber Pakhtunkhwa-Pakistan.

2.4. Preparation of spore suspension

Potato dextrose agar (PDA) slants (Oxide, USA) were applied for preserving the mould cultures for seven days at 25°C. The mould spores were collected from the sporulating colonies, shifted into sterilized distilled H_2O holding 0.1%v/v tween 80 and poured into autoclaved distilled H_2O bottle. The spore's numbers were calculated using Neubrour Haemocytometer- BDH Ltd., Dagenham, England (Reddy *et al*, 2009).

2.5. Efficacy of NSC on the growth of Fungi

Take sterilized poultry feed (30g) and added a known quantity of NSC (0.5-20w/w) in an autoclaved Petri dishes. A 0.1mL ($25x10^7$ CFU/mL) dilute spore suspension of experimental mould were poured to the center of Petri plates holding sterilized poultry feed and incubated for 6 weeks at room temperature. The poultry feed without NSC was applied as control. The incubated plates were then seen for fungal growth weekly (Thanaboripat *et al*, 1997). The decontaminated feed without mould inoculation and NSC served as negative control (growth score=0), while decontaminated feed seeded with mould and without NSC represent as the positive control (growth score=5).

2.5.1 Observation of fungal growth

Inhibition of fungal growth in poultry feed were assessed visually by scale.

0=No growth.
1=Very little growth.
2=25% of feed covered.
3=50% of feed covered.
4=75% of feed covered.
5= 100% of feed covered (Cuero *et al*, 1987).

2.5.2 Spore count

The spores of experimental mould were collected from poultry feed samples with distilled water (sterilize) holding Tween 80 (0.1%). The suspension of spores was filtered and the quantity of spores were measured applying haemocytometer. The sieve spore suspension 9µL was reserved and shifted to one of the measuring 4 corner of chamber. The spore suspension was released gradually to evade overfilling and bubble. The spores were then measured in each of the 0.1 mm^3 square.

Quantity of spores were measured through utilizing the equation; Spores/ml= (n) $x10^4$

Wherever, n = The average colony amounts per square of the 4 corner squares measured (Thanaboripat *et al*, 1997).

2.6. Feed Sample Treatment

Toxin free and fresh poultry feed samples were collected from Feed Shops retailer in vicinity of Peshawar, Khyber Pakhtunkhwa-Pakistan. The collected samples were brought into Laboratory, dried at 60°C in an oven (Memmert Germany, Ambient-250 ºC) and divided into 200g parts as per experimental design. Each portion was sterilized, after that soaked (16%) with distilled H_2O (sterilized). The poultry feed samples were seed through 4mL of *A. parasiticus* and *A. flavus* suspension (1×10^7 spores /mL) under Vertical Laminar Flow Cabinet (Streamline Laboratory Products, Maxwell Road Singapore). The dosage of NSC (01, 5, 10, 15 and 20% w/w) were blended separately in 200g of inoculated poultry feed. Un-treated poultry feed samples were measured as control. The treated and un-treated experimental samples were kept at 28 °C and 16% moisture for 6 months. At the close of every month, poultry feed samples were drawn and AFs (G2, G1, B1 and B2) were measured using TLC method.

2.6.1 Extraction of AFs

The AFs extraction was carried out applying the procedure (Pons *et al*, 1966). A poultry feed sample of 50gm was taken in a 500mL *Erlenmeyer flask and extracted with 70% aqueous acetone (250mL) for 1 hour using a* horizontal shaker. The mixture was filtered and the filtrate were cooled. The amount of the filtrate was minimized to 140mL on a hot water bath (Colra, Germany, Ambient ~ 100 ºC). A bowl of distilled water and 20mL of lead acetate were added after cooling. The mixture was filtered using Whatman No.1 filter paper and the filtrate were centrifuge for 10 minutes at 10,000 rpm. The achieved supernatant was extracted in a separating funnel with 50mL of chloroform. The layer of chloroform was obtained passed through sodium sulphate (anhydrous). The achievable liquid layer was evaporated to dryness to gain the aflatoxins crude extract.

2.6.2 Estimation of Aflatoxin by Thin Layer Chromatography

The prepared TLC plate (20x20cm), Silica gel 60 F_{254} (Merck-Damstadt-Germany) were used and the AFs standard and samples were spotted on plates with the help of micropipettes. The chromatography was developed in a chromatography tank holding toluene, ethyl acetate and formic acid (60:30:10) to the depth of <1cm. The solvent was permitted to run for 10 to 12cm. The plates were dried in parallel position and observed under UV Viewing Cabinets (CV-00612 Lamps Cambridge England). The Rf value were checked AFs standards with the sample. The occurrence of blue fluorescent spot was corresponding to AFs (B) at Rf 0.80-0.67, while the green florescence spot corresponded to AFs (G) at Rf values ranging from 0.59-0.48. The AFs content were measured (AOAC, 1980; Pons *et al*,1966) using the following formula.

$$\textbf{Aflatoxins (µg/kg)} = \frac{S \times Y \times V}{W \times X}$$

where

S is the µL AFs standard which matched the unknown
Y is the concentration of standard AFs in µg/mL extract
V is the µL of solvent required for final dilution of sample extract
X is the µL of sample extract spotting giving fluorescent intensity equivalent to S (Standard)
W is the Weight in gm of original sample hold in the ending extract

% inhibition of AFs was measured as follows:

$$\textbf{\% inhibition} = \frac{\text{AFs level in Control- Treated}}{\text{AFs level in Control}} \times 100$$

3. Statistical analysis

All experimentations were completed in triplicate (n=3) and the results therefore gained were described as means ± SD. Analysis of variance (ANOVA) was performed at $P < 0.05$ confidence level using Minitab statistical software (Minitab Inc. Pennsylvania, U.S.A).

4. Results

The neem seed cake residue powder FTIR analysis is shown in Fig.1. The FTIR spectroscopic screening of the NSC displayed a total of eighteen different absorption peaks, indicating the occurrence of a diversity of functional groups linked with organic substances. The prominent peaks were showed at 3294.42, 2916.37, 2858.51, 2395.59, 1745.58, 1645.28, 1541.12, 1448.54, 1384.89, 1338.60, 1242.61, 1159.22, 1109.07, 1033.85, 860.25, 813.96, 671.23, and 597.93 cm^{-1}. The powerful and broad band around 3294.42 cm^{-1} corresponds to O–H stretching vibrations OH groups, characteristically occurred in phenolic and alcoholic compounds. Peaks at 2916.37 cm^{-1} and 2858.51 cm^{-1} are accredited to C–H stretching of aliphatic $–CH_2$ and $–CH_3$ groups. The absorption peak near 1745.58 cm^{-1} displayed the occurrence of C=O stretching of ester or carbonyl molecules, whereas the peaks at 1645.28 cm^{-1} suggests C=C stretching of alkenes or aromatic rings. The bands displayed at 1541.12 cm^{-1} and 1448.54 cm^{-1} correspond to N–H bending and C–H deformation vibrations, respectively. Absorption in the region 1384.89–1033.85 cm^{-1} signify C–N stretching of amines and C–O stretching of carboxylic acids, esters and alcohols. The peaks between 860.25–597.93 cm^{-1} are linked with C–H out-of-plane bending and C–Cl stretching, approving the complex organic nature of the neem seed residue. Generally, the FTIR peaks authorize the occurrence of functional groups like amine, alkene, carbonyl and hydroxyl, which accredited to the bioactive and biochemical activities of neem seed cake residue. These functional groups are recognized to perform significant role in antifungal and antibacterial properties, displaying the potential of neem seed cake as natural or bioactive antifungal substance.

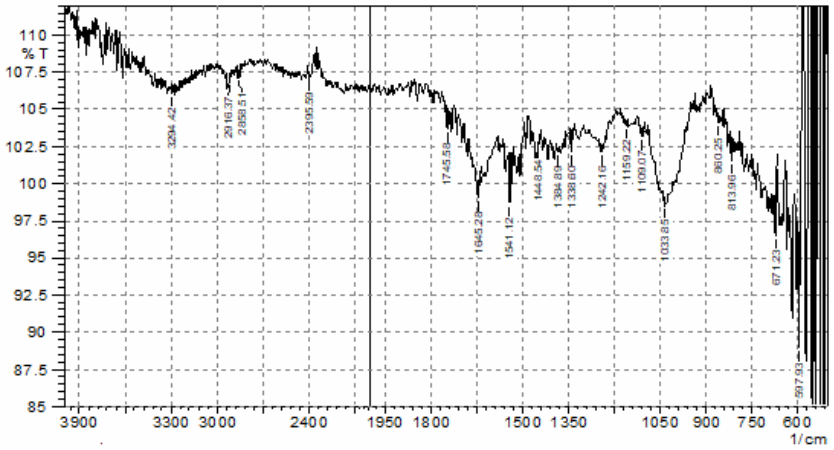

Fig. 1. FTIR of NSC

The effect of NCS on the growth of *A. flavus* in poultry feed during 6 weeks of incubation is shown in Table 1. No mould growth was showed in the negative control throughout the experiment, however the positive control displayed complete feed

colonization (score 5) from the first week ahead. The NSC significantly ($P < 0.05$) suppressed the growth of A. flavus in a dose- and time-dependent way. The minimum dosage (1.0% w/w) permitted progressive mould growth, achieving whole colonization through week 6, and did not diverge significantly from the positive control ($P > 0.05$). In dissimilarity, NSC at 5% and 10% (w/w) significantly minimized mould growth ($P < 0.05$), with highest growth scores of 4 and 3, respectively. Noticeable suppression was achieved at maximum NSC dosage (15% and 20% w/w), wherever mould growth persisted nominal throughout the storage time and diverged significantly from the positive control ($P < 0.05$). The maximum antifungal activities were observed at 20% NSC, which steadily restricted mould growth to very small levels (score ≤ 1).

Table 1. Effect of Neem Seed Cake on the growth of *A. flavus* in Poultry Feed.

Incubation Time	-ve Control	+ve Control	% Concentration (w/w)				
			1.0	5	10	15	20
	*Score observed						
1st week	0	5	1	1	1	0	0
2nd week	0	5	2	1	1	1	1
3rd week	0	5	2	2	1	1	1
4th week	0	5	3	2	2	1	1
5th week	0	5	4	3	3	2	1
6th week	0	5	5	4	3	2	1

***** Growth scores were assigned as described in the Materials and Methods.

Table 2 is summarized the effect of NSC on the *A. parasiticus* growth in poultry feed over a six-week incubation time. No mould growth was showed in negative control, however the positive control observed whole feed colonization (score 5) all over the experiments. The NSC significantly minimized ($P < 0.05$) the growth of *A. parasiticus* in a dose-dependent way. At 1.0% (w/w), mould development raised steadily over storage, attainment a score of 3 week six and lasting significantly lesser than the positive control $P < 0.05$). Moderate controlling was displayed at 5% and 10% (w/w), with growth score not greater than 4 and 3 respectively. Maximum NSC dose (15% and 20% w/w) displayed marked antifungal activities, keeping minimum mould growth throughout the incubation time. The scores of growths at these doses remained ≤2 and were significantly minimum than those of the positive control ($P < 0.05$). The highest inhibitory effect was showed at 20% NSC, wherever mould growth persisted very restricted during the whole storage time. The incubated poultry feed without mould and NSC (negative control) was observe clear and score 0 whereas poultry feed holding mould without NSC (positive control) was completely shielded by mycelium and scored 5.

Table 2. Effect of Neem Seed Cake on the growth of *A. parasiticus* in Poultry Feed.

Incubation Time	-ve Control	+ve Control	% Concentration (w/w)				
			1.0	5	10	15	20
	*Score observed						
1st week	0	5	2	3	2	0	0
2nd week	0	5	2	3	2	1	1
3rd week	0	5	2	3	2	1	1
4th week	0	5	2	3	2	2	1
5th week	0	5	3	4	3	2	1
6th week	0	5	3	4	3	2	2

*****Growth scores were assigned as described in the Materials and Methods.

The effect of NSC powder on mould spore amounts in poultry feed after 6 weeks of storage is shown in Table 3. The blanks samples displayed the maximum spore loads for both *A. parasiticus* (452 spores/g) and *A. flavus* (340 spores/g), verifying widespread mould growth in untreated feed. Addition of NSC significantly decrease ($P < 0.05$) the spores' number in dose-dependent way for both mould species. At 1.0% (w/w), the count of spores was decreased to 190 spores/g for *A. flavus* and 287 spores/g for *A. parasiticus,* lasting significantly lesser as compared with control ($P < 0.05$). Additional decreases at 5% and 10% NSC, with spores amounts decreasing suddenly for both moulds. Noticeable inhibition of sporulation was observed at maximum NSC dosages (15% and 20% w/w). At 20% NSC, spore amounts were decreased to 15spores/g for *A. parasiticus* and 10spores/g for *A. flavus* representing the powerful antifungal efficiency and observing significant variations as matched to the whole lesser dosages and control ($P < 0.05$). Generally, powdered NSC on AFs synthesis inhibited the mould growth sporulation in poultry feed, with *A. parasiticus* displaying slightly maximum spore counts as compared with A. flavus across all treatment.

Table 3. No. of spores of Fungi in poultry feed (spores/g) at the end of 6th week.

Tested Fungi	Number of spores/grams					Control
	Percent concentration of powdered NSC (w/w)					
	1.0	05	10	15	20	
Aspergillus Flavus	190±0.3	140±0.4	50±0.1	20±0.2	10±0.3	340±01
Aspergillus parasiticus	287±0.5	230±0.8	60±0.2	30±0.1	15±0.5	452±01

Values were performed in triplicate and represented as the mean ± Standard deviation (SD).

The effect of NSC on AFs production by *A. flavus* in poultry feed during 6 months of incubation is illustrated in Table 4. In untreated control samples, AFs levels augmented gradually over period, attainment 50 µg/kg for AFB2 and 80 µg/kg for AFB1 by the 6 months. Treatment with NSC significantly minimized ($P < 0.05$) AFs creation in a concentration- and period-dependent way. For AFB1, the minimum NSC (1.0% w/w) resulted in modest suppression, however maximum dose (10–20% w/w) manufactured significant suppression throughout the storage time. The maximum suppression activity was showed at 20% NSC, where AFB1 level keep on ≤22 µg/kg after 6 months, conforming to a highest suppression of 72% as matched to the control. A parallel pattern was detected for AFB2 synthesis. The NSC blends significantly suppressed ($P < 0.05$) AFs level linked to control, with powerful suppression at maximum doses. The NSC 20%, the AFB2 level were condensed to 18 µg/kg by the 6 months, representative up to 64% suppression. At initial storage phases, AFB2 was not present or remained at very small degree in NSC-treated samples, especially at dosage ≥10% (w/w). Generally, NSC efficiently inhibited AFs growth in poultry feed, with maximum dosages (15–20% w/w) delivered continued and statistically significant ($P < 0.05$) inhibition of both AFB2 and AFB1 throughout the incubation time.

Table 4. AFs (µg/kg) content in Poultry Feed Treated with NSC against *A. flavus*.

Treatment	Dosage (%)	1st month	2nd month	3rd month	4th month	5th month	6th month
Control	00	15±0.1	24±0.5	32±0.2	45±0.4	65±0.1	80±0.6
AFB1	1.0	14± 0.1 (07)	20 ± 0.5 (17)	26 ± 0.3 (19)	35± 0.1 (22)	50± 0.1 (23)	60±0.5 (25)
	5.0	13± 0.2 (13)	19± 0.5 (21)	24± 0.8 (25)	30± 0.5 (33)	42± 0.1 (35)	49± 0.1 (39)
	10	10 ± 0.5 (33)	11 ± 0.1 (54)	15± 0.5 (53)	17 ± 0.2 (62)	26 ± 0.4 (60)	35 ± 0 (56)
	15	07 ± 0.3 (53)	08 ± 0 (67)	09± 0 (72)	12 ± 0 (73)	18 ± 0 (72)	28 ± 0.5 (65)
	20	02± 0.1 (87)	03 ± 0 (87)	04± 0 (87)	09 ± 0 (80)	12 ±0 (81)	22 ± 0.5 (72)
Control	00	09±00	10±00	14±0.1	31±01	43±0.5	50±0.4
AFB2	1.0	ND	ND	12± 0.5 (14)	26±0.1 (16)	35± 0.4 (18)	40± 0.1 (20)
	5.0	07± 0.5 (22)	07± 0.1 (30)	10± 0.1 (29)	22 ± 0.1 (29)	30±0.1 (30)	34± 0.3 (32)
	10	06 ±0 (33)	07 ±0 (30)	08± 0(43)	15± 0.5 (52)	17 ± 0 (60)	28 ±0.5 (44)
	15	05 ± 0 (44)	06±0 (40)	07± 0 (50)	11± 0.5 (64)	13 ± 0 (70)	20 ± 0.4 (60)
	20	04 ± 0(56)	05± 0 (50)	06±0 (57)	10 ± 0 (68)	11 ± 0 (74)	18 ± 0.8 (64)

All values represent mean ± standard deviation and were rounded to the nearest whole number. Values in parentheses represent inhibitory effect (%) of NSC as compared to control.

The effect of NSC on AFs production by *A. parasiticus* in poultry feed over six months of storage summarizes is shown in Table 5. In untreated controls, the quantification of AFG2, AFG1, AFb2 and AFB1 raised gradually with time, reaching 38, 68, 60 and 105 µg/kg, respectively, the six months. The NSC significantly inhibited ($P < 0.05$) the synthesis of all 4 AFs in dose- and time-dependent way. At minimum dose (1.0–5.0% w/w), only modest decreases were showed, while maximum dose (10–20% w/w) outcomes in noticeable embarrassment throughout the storage time. The maximum decrease in AFB1 was observed at 20% NSC, wherever AFs level were minimized to 35 µg/kg at six months, consistent to 67% suppression matched to the control. A comparable suppression outline was displayed for AFG2, AFG1, AFB2 and AFB1. The NSC 20%, the maximum hang-up values of AFB2 (85%), AFG1 (75%) and AFG2 (76%) were reached through the six months, all of which were pointedly lower than the control and lower NSC treatments ($P < 0.05$). Generally, NSC at dosage of 15–20% (w/w) delivered sustained and statistically significant inhibition of AFs buildup in poultry feed seeded with *A. parasiticus*.

5. Discussion

5.1. Bioactive Compounds of Neem

The FTIR spectrum was applied to recognized the functional groups of the bioactive compounds' origin on the peak value in the area of infrared radiation. The carboxylic acids have an alcohol and carbonyl group they share some fundamental chemical and physical characteristics with alcohols, ketones and aldehydes (DeRuiter, 2005). The carboxylic acid esters were evaluated *In vitro* against Aspergillus niger, candida albicans and *Cryptococcus neoformans.* All carboxylic acid ester derivatives hold maximum

antifungal properties as compared with fluconazole (Nam *et al*, 2004). The aldehyde is an organic substance holding a formyl group. Amongst the aldehydes, glutaraldehyde is a significant dialdehyde utilized as disinfectant and sterilant. Glutaraldehyde has a wide-ranging of activities against viruses, fungi and bacteria. Cinnamaldehyde and formaldehyde are also applied as fungicides (Sadiqullah *et al*, 2018). A great number of various nitriles have been acaricidal and insecticidal activities. The halogenated aromatic dinitriles displayed excellent biological activities as nematocides, bactericides and fungicides (Melnikov *et al*, 2012).

The FTIR of de-oiled seed cake of neem, which mostly comprise of aromatic and aliphatic substances. The aliphatic comprise alkynes, alkenes and alkanes. The C–H stretching at 2853 and 2923 and C-H bending at 1240 designate the occurrence of alkanes. The C=C Stretching at 1639 specify the occurrence of alkenes. The C=C Stretching at 1450 and 1544 bending at 609 and 832 showed aromatic substances in neem de-oiled sed cake. The marked oxygenated functional groups O-H, C=O, C-O and aromatic substances designate that the de-oiled neem seed cake was vastly hydro oxygenated and thus, its nature is acidic. The oxygenated molecules decrease the calorific degree of de-oiled neem seed cake (Muliman and Navindgi, 2016).

The FTIR peaks analysis of *Azadirachta indica* (neem) seed cake residue in the current study exposed typical absorption peaks agreeing to significant functional groups like amine (–NH) moieties, alkene (C=C), carbonyl (C=O) and hydroxyl (–OH). These results are in close promise with the previously documented plant-based extracts. For example, similar broad –OH stretching peaks (~3200–3400 cm^{-1}) and C=O stretching vibrations (~1650–1750 cm^{-1}) were showed in the extracts of *Ocimum basilicum* (Zahoor *et al*., 2026), specifying the occurrence of flavonoids and phenolic compounds. Similarly, the leaves extract of *Melia azedarach* (Ali *et al*., 2025a) showed the analogous spectra linked with secondary metabolites, proteins and lignin, proofing the structural resemblance among Meliaceae species. Moreover, the FTIR peaks documented for neem twigs, leaves and seeds extracts prepared though microwave, decoction and infusion techniques (Ali *et al*., 2023) also established steady functional groups signs, predominantly in the fingerprint area (1000–1500 cm^{-1}), which is accredited to aromatic and polysaccharides compounds.

However, slight variations in peak intensity and shifting were observed in the current study, which may be attributed to differences in sample matrix (seed cake residue vs. fresh plant material), extraction techniques, and the presence of residual oil fractions. These distinctions highlight the uniqueness of neem seed cake residue as a value-added byproduct with retained bioactive functional groups. Overall, the comparative FTIR analysis confirms that neem seed cake residue possesses chemically active constituents similar to other medicinal plant extracts, reinforcing its potential application in antifungal and aflatoxin inhibition studies.

The extracts of neem are comprising of antimicrobial substances like alkaloids, glycosides, flavonoids, saponins and phenols, which are common antibiotics phytochemicals founds in plants (Pandey *et al*, 2014; Ali *et al*, 2022; Asif, 2012; Anokwuru *et al*, 2011). The quercetin and ß-sitosterol (1st polyphenol flavonoids) isolated from neem fresh leaves, are reported to holds fungicide and bacteriostatic properties (Mahmoud *et al*, 2011). The purified compounds from neem have been classified into two groups: (1) isoprenoids which consists triterpenoids and diterpenoids comprising azadirone, protomeliacins, limonoids and comprise derivatives, gedunin and its derivatives, vilasinin type of substances and C-secomeliacins like azadirachtin, nimbin and salanin. (2) Non- isoprenoids, which are polyphenols (coumarin, flavonoids, dihydrochalcone, glycosides and tannins), aliphatic substances, Sulphur substances, proteins, carbohydrates etc. The widely held of these ingredients have fungicidal properties (Asif, 2012).

The neem bark, seeds and leaves are documented as treasured origins of vital nutrients such as minerals, carbohydrates, fiber, fats and proteins, donating meaningfully to their overall energy contented. Because of its ridiculous contour, neem-derived products have been combined into dietary supplements, animal feed and food formulations to address undernourishment. Their application is especially pertinent in underprivileged and developing regions, where they can act as economical substitutes to conservative nutritious supplements. Furthermore, the application of neem-origin products in poultry, livestock and fish feed can decrease requirement on imported feed additive from developed countries, thus saving foreign currency and aiding homegrown financial growth via occupation creation (Ali *et al*., 2025b).

5.2. Antifungal Activities of Neem

At 10% dosage neem oil provided 100% inhibition growth of moulds viz *Macrophomina phaseolina, Drechslera rostrate, Aspergillus niger* and *Fusarium moniliforme* (Vir and Sharma, 1985). It was documented that 2-10% neem oil completely controlled the growth of *Fusarium oxysporum, Aspergillus niger and Alternaria alternata* (Locke, 1995). It was reported that neem oil moderately effective against Aspergillus species (Niaz and Kazmi, 2005). The *Brassica campestris* (mustard) and *Azadirachta indica* have documented antifungal activities against eight pathogenic fungi *Drechslera hawiensis, Alternaria alternate, Fusarium semitectum, F nivale, F oxysporum, Fusarium moniliforme, Aspergillus niger* and *A. flavus* (Sitara *et al*, 2008). The neem seed oil antifungal action has been reported against soil borne fungi (Locke, 1986; Singh *et al*, 1980a). The neem seed oil antifungal activities have been accredited to its sulfur composition (Singh *et al*, 1980b). While using maximum dosage of seed oil of neem might additional improve its fungicidal properties, dosage >2% leave gummy precipitate on the fruit surface that could decrease customer reception (Harold and James, 1993). The neem oil (crude) was observed maximum fungicidal properties as matched to pure fractions originated through solvent partitioning (Govindachari *et al,* 1998).

The neem leaves extract ethanol, methanol, chloroform and ethyl acetate at 75mg/mL inhibited 84.1%, 81.8%, 63.6% and 56.8% of the mycelium dry weight of *A. flavus* respectively (Ali *et al*., 2026). The neem seed oil at 50 µL/mL concentration complete inhibition of mould. The neem seed oil at 50 µL/mL was reported to be fungicidal. The neem seed oil MIC was documented to be 10µL/mL at which dosage spore's revival were seen later inoculating on fresh PDA in 7 days. Like findings were also observed in case of *A. parasiticus* through the utilization of neem seed oil. The water extracts at 50mg/mL dosage no effect was observed against *A. parasiticus* and *A. flavus* (Faraz *et al*, 2005). The current and the previous study (Ali *et al*., 2026 and Faraz *et al*., 2005)

findings are a close agreement in term of aflatoxin inhibition level, but the neem leaves and neem oil used at very low level, but in our study the neem seed cake residue is used in a high concentration to inhibit the toxigenic moulds and inhibited the aflatoxin. The neem seed extracts (NSE) potentially inhibited the mycelium biomass *A. flavus* and *A. parasiticus* in dose-dependent manner. The whole embracement of mycelium development of treated fungi was exhibited at 500mg/mL (Javid *et al*., 2026). The seed kernel of neem is very wealthy in fatty acids, often up to fifty percent of the weight of kernel. Seed oil of neem is fairly bitter with a sulfur/garlic smell and holds essential amino acids and vitamin E. Previous findings showed that neem leaves might suppress the growth of Aspergillus (Bhatnager and McComick 1988; Ali *et al*, 2023). The neem leaves extract of ethanol, methanol, chloroform and ethyl acetate observed biomass inhibition of *A. parasiticus* were 86.1%, 82.6%, 72.2% and 60.6% respectively (Ali *et al*., 2026). The neem seed powder was applied at dosage of 10%, 5.0%, 2.0%, 1.0%, 0.5% and 0.1% were found to be active against *Rhizopus species, Aspergillus flavus* and *Aspergillus niger* (Dauda *et al*, 2015). The water neem seed and neem extracts minimize the fungi growth predominantly *Pyricularia oryzae* on rice (Amadioha, 1999). The current study showed a dose dependent antifungal activity against the tested moulds, these finding is in a clos agreement of (Ojo *et al*, 2011; Chigoziri and Ocholongwa, 2017) which described that the suppression activities of extracts on the growth of mycelia raised with raised the dosage. Consequently, the efficiency was extremely subtle to neem seed powder is a very wanted progress, all that is wanted to suppressed of *Rhizopus* species from the seed of groundnut using neem seed powder (5%) which is a very small and will be very cost-effective (Dauda *et al*, 2015). In the current study the neem seed cake residue is also active at 5% dose, but inhibition of aflatoxin degree is low and at higher dose 15% and 20% the aflatoxin inhibition was increases, because in our study we used the neem seed cake residue mean the oil is extracted form the seed and the residue is left, which is a by-product of neem oil extraction unit.

The maximum antifungal activities were found at 500mg/mL for the fungal species, observing 85% and 83% spore's germination inhibition for *A. parasiticus* and *A. flavus* respectively noteworthy. These results proposed that leaves aqueous extract of *Melia azedarach* showed a significant anti-mould activities, with rising concentration of the extracts increased the antifungal activities (Ali *et al*., 2025a). The chemicals substances, benzoic acid (0.1–0.5%), copper sulphate (0.08–0.5%), ammonia (0.5%) and propionic acid (0.1–0.5%) totally suppressed the growth of *A. parasiticus*. The sodium propionate, citric acid and urea had moderate antifungal activities (36–64%) reduction (Gowda *et al*, 2004). The herbal and plant based antifungal compounds have some advantages such as biodegradable in nature, non-toxic to human and animal, ecofriendly, easily available and cost effective. So, the herbal substances such as clove oil at 0.5% totally suppressed the mould growth (Gowda *et al*, 2004). While the neem seed and leaves extracts have antifungal properties against post-harvest fruits rot mould (Ali *et al*, 2025c). The neem root and flower aqueous and ethanolic extracts were evaluated against post-harvest pathogenic mould i.e. *Monilinia fructicola* and *Rhizopus stolonifer* (Javid *et al*, 2025). The minimum fungicidal dosage of twigs, seeds and leaves of ethyl acetate, hexane, chloroform, ethanol and methanol against *Penicillium expansum, Colletotrichum gloeosporioides* and *Botrytis cinerea* were in the range of 62.5 to 1000 mg/mL (Ali *et al*, 2025d).

The inhibition effects of pongamia cake, neem cake, peels powder (*Punica granatum, Citrus medica* and *Citrus sinensis*), leaves powder (*Eucalyptus citriodora, Hyptis suaveolens* and *Withania somnifera*), Captan and cowdung fumes and spores' suspension of Aspergillus niger and Trichoderma harzianum were evaluated on aflatoxin B1 biosynthesis through the isolated toxigenic strain of *A. flavus*. The findings observed that all the treated experiments were significantly inhibited aflatoxin B1 biosynthesis. Spores suspension of *Aspergillus niger*, *Trichoderma harzianum* and both, neem cake and Captan declined the quantities of B1 (aflatoxin) to a maximum level (Shashikala and Krishnamurthy, 2006).

5.3. Inhibition of Aflatoxin Biosynthesis

It is documented that plants produce a numerous bioactive substance in plants tissues such as saponins, terpenoids, tannins, flavonoids, alkaloids and other substances, described to holds in vitro antifungal activities (Arif *et al*, 2009). Total inhibition (100%) observed for all four types aflatoxins (G1G2, B2, B1) synthesized by *A. parasiticus* and for both aflatoxins (B2 and B) synthesized through *A. flavus* at highest concentration i.e. 500mg/mL in all extracts (Javid *et al*., 2026). It stated that the fungal cell wall is the chief target of neem compounds. The cell wall of fungi is vigorous to defend the cell from osmotic pressure and the trouble in the cellular osmotic pressure could rise cell vacuolation or even decease of the microbes through osmotic tremor (Sazykin *et al*, 1995). The observation outcomes regarding morphology changes were reported the cell wall obliteration accredited to other antifungal compounds (Joklik and Mitchell, 1980).

The neem leaves ethanol extract displayed aflatoxin B1 inhibition was 90.7% in *A. parasiticus* and 89.3% in *A. flavus* and methanol (82.4% and 81.3%), chloroform (68.5% and 63.4%) and ethyl acetate (62.2% and 57.1%) (Ali *et al*., 2026). Leaves flour extract of neem have the capability to detoxify ochratoxin A and aflatoxin B1 when mixed to rice, beans, maize and wheat for long-term stowage, and it might bound the growth of *A. parasiticus* and *A. niger* (Mir *et al*, 2021). The findings confirmed that neem seed powder (NSP), especially at high concentration (20%–30%), was particularly effective in preventing or suppression aflatoxins in poultry feed stored over for a six month, even when treated with aflatoxin producing moulds (Javid *et al*., 206). In the current study it is also demonstrated that at 15% and 20% concentration the aflatoxin inhibition rate increases and it is a close agreement of the previous study (Javid *et al*., 2026). By blending herbal extracts from *A. indica* tree in processed animal feed, in this case, soybean pellets, therefore minimizing the danger in controlling mycotoxin adulteration. The extract of neem is known to have suppression effects on moulds which able to synthesizing mycotoxin, from AFs strains. In this research, neem extract is blended at 20% (v/v) in the soybean treated into pellets (Ida *et al*, 2014). It has been reported that the neem leaves extract addition at a dosage higher than 10% (v/v) effectively suppressed AFs synthesis (Bhatnagar and Mccormick, 1988). Neem extracts affects

with the metabolic pathways of AFs production without interfering mould growth (Zeringue and Bhatnagar, 1990). The neem extract at a dosage of 50% (v/v) caused more than 90% suppression in AFs synthesis (Zeringue and Bhatnagar, 1990). Subsequent these examinations, it is further reported that mould growth is inhibited which is linked to mould cell wall demolition, according to its morphological outcomes (Abyaneh *et al*, 2003). The report of (NRC, 1992) stated that the neem leaves extract completely suppressed the biosynthesis of AFs produced through *A. flavus* on cotton seed. The Ocimum basilicum seed extracts of alcohol were the most efficient, controlling spore germination of *A. flavus* and *A. parasiticus* by 85% and 80% at a dose of 250mg/mL and declining aflatoxin biosynthesis by 85% (Zahoor *et al*., 2026). The difference between the current findings and the study of (Zahoor *et al*., 2026) accounted that the dissimilates of plants nature, types, parts, concentration, experimental procedures, extraction procedure, solvents types, extracts.

Table 5. AFs (µg/kg) content in Poultry Feed Treated with NSC against *A. parasiticus*.

Treatment	Conc. (%)	1st month	2nd month	3rd month	4th month	5th month	6th month
Control	00	19±0.1	29±0.5	43±0.6	61±0.5	74±0.8	105±02
AFB1	1.0	18± 0.1 (05)	27± 0.5 (07)	38±0.4 (12)	52± 0.6 (15)	62± 0.7 (16)	87± 05 (17)
	5.0	17± 0.1 (10)	25± 0.5 (14)	36± 0.8 (16)	50± 0.6 (18)	59± 0.5 (20)	83± 0.5 (21)
	10	14 ± 0 (26)	17 ± 0 (41)	18 ± 0.5 (58)	24 ± 0.6 (61)	30 ± 0.2 (59)	42 ± 0 (60)
	15	10 ± 0 (47)	16 ± 0 (45)	17 ± 0.3 (60)	22 ± 0.2 (64)	27 ± 0.6 (63)	39 ± 0.6 (63)
	20	05 ± 0 (74)	10 ± 0 (65)	16 ± 0.2 (63)	21 ± 0.5 (66)	26 ± 0.7 (66)	35 ±0.4 (67)
Control	00	13±0.1	19±0.5	26±0.6	41±0.5	50±0.5	60±0.5
AFB2	1.0	12± 0.1 (08)	17± 0.5 (10)	23± 0.5 (11)	36± 0.6 (12)	43± 0.7 (14)	51±0.5 (15)
	5.0	11± 0.1 (15)	16± 0.2 (16)	21± 0.5 (19)	32± 0.4 (22)	38± 0.5 (24)	45± 0.5 (25)
	10	10 ±0 (23)	11 ± 0 (42)	12 ±0 (54)	15± 0 (63)	14± 0 (72)	15 ± 0 (75)
	15	5 ± 0 (61)	08 ±0 (58)	11 ± 0 (58)	13± 0 (68)	16 ± 0 (68)	14 ± 0 (77)
	20	4 ± 0 (69)	03 ± 0 (84)	05 ± 0 (81)	07± 0 (83)	08 ± 0 (84)	09 ± 0 (85)
Control	00	14±0.1	18±0.5	27±0.4	38±0.5	54±0.2	68±0.5
AFG1	1.0	13±0.1 (07)	16± 0.5 (11)	23±0.5 (15)	32± 0.5 (16)	45 ± 0.5 (17)	55± 0.5 (19)
	5.0	12± 0.1 (14)	15± 0.5 (17)	22 ± 0.2 (18)	31± 0.2 (18)	44± 0.5 (18)	54±0.5 (21)
	10	11± 0 (21)	12 ± 0 (33)	17 ± 0 (37)	23 ± 0 (39)	30 ± 0 (44)	35 ± 0 (48)
	15	09 ±0 (35	11 ± 0 (39)	16 ± 0 (41)	21 ± 0 (45)	27 ± 0 (50)	33 ± 0 (51)
	20	04 ± 0 (71)	05 ± 0 (72)	07 ± 0 (74)	10 ± 0 (74)	14 ± 0 (74)	17 ±0 (75)
Control	00	15±0.1	20±0.4	25±0.5	31±0.1	36±0.5	38±0.4
AFG2	1.0	14± 0.1 (07)	18± 0.2 (10)	22± 0.5 (12)	27 ± 0.5 (13)	31± 0.1 (14)	32± 0.1 (16)
	5.0	13± 0.1 (13)	17± 0.5 (15)	21± 0.1 (16)	25± 00 (19)	28± 00 (22)	29± 00 (24)
	10	12 ± 0 (20)	13 ± 0 (35)	14 ±0 (44)	15 ± 0 (52)	16 ± 0 (56)	16 ± 0 (58)
	15	08 ± 0 (47)	09 ± 0 (55)	11 ± 0 (56)	13 ± 0 (58)	14 ± 0 (61)	14 ± 0 (63)
	20	05 ± 0 (67)	06 ± 0 (70)	07 ± 0 (72)	08 ± 0 (74)	09 ± 0 (75)	09 ± 0 (76)

All values represent mean ± standard deviation and were rounded to the nearest whole number. Values in parentheses represent inhibitory effect (%) of NSC as compared to control.

Azadirachta indica can also attend as a vital origin of sheep, cattle and poultry feed. Seed extracted materials (Mitra, 1963), when utilized as mixtures of feed for numerous laying poultry, have been compatible with peanut as a food stuff (Gupta *et al*, 1975). Likewise, processed A. indica seed meal rich in protein and free from lipid and fat connections (Mitra and Misra, 1967) was edible to buffalo at 25% dosage (Bedi *et al*, 1975). Neem seed cake ration was edible at the 10-20% quantity without any effect on milk ingredients during lactation (Pyne *et al*, 1979) or on white and red blood cell counts or on red and white blood cell counts (Gangopadhyay *et al*, 1979). In circumstance, a steady rise of neem cake feed improves the P and Ca quantity of blood (Gangopadhyay *et al*, 1981). The neem cake (de-oiled) could be combined up to 25-50% in Zea mays diet for sheep without poisonous effects (Gupta and Bhaid, 1981).

The synergetic action of the recognized bioactive substances in the neem leaves extract is accountable for its multi-layered profits such as hepatoprotective benefits, better intestinal morphology, better feed conversion ratio and body weight, minimized the heterophil-to-lymphocyte ratio and amplified lymphocyte counts, it a cost-effective, natural and promising phytogenic substitute to in-feed antibiotics to improve production and confirming harmless broiler meat (Shafiqul *et al*., 2026). The nutritional supplementation of neem leaves extracts pointedly enhanced feed conversion ratio, growth performance and precise growth rate of *Labeo rohita* fingerlings. Among the experimental trails, one percent neem blend produces the most auspicious outcomes. Neem leaves extracts might thus be measured an effective, non-synthetic feed preservative for sustainable aquaculture (Saleha *et al*., 2026).

The current study determines an inimitable incorporation of antifungal activities, FTIR-based functional profiling and aflatoxin production inhibition applying neem seed cake residue. Contrasting previous findings that mostly focus on either antifungal

activities or toxin adsorption, this research highpoints a blend mechanism linking both spore's inhibition, mould growth suppression and inhibition of toxin synthesis. Additionally, the application of neem seed cake residue signifies a value-added utilization of an agro-food by-products, improving its applied significance for large-scale feed sanitization.

6. Conclusion

The results of this study reported that NSC is an efficient natural antifungal and anti-aflatoxigenic substance in poultry feed. The NSC significantly suppressed the growth and sporulation of *A. parasiticus* and *A. flavus* in a dose-dependent way, with maximum dosage (15–20% w/w) delivered the powerful and most reliable inhibition over storage. Near-complete or complete control of mould growth was displayed at higher NSC levels, go with through a noticeable decrease in spore production. Moreover, NSC noteworthy minimized the buildup of AFs (G2, G1, B2 and B1) during incubation, with suppression raised with both NSC dosage and storage period. The maximum NSC dosage (20% w/w) findings in constant decrease of up to 70–85% in AFs level matched to untreated controls. Generally, the outcomes suggested that NSC has powerful properties as eco-friendly, cost-effective and safe feed additive for inhibition mould contamination and reduction AFs risks in poultry feed during storage.

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