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COMPARATIVE RESPONSE OF BROWN RICE GENOTYPES TO INDIGENOUS PLANT EXTRACTS AGAINST ASPERGILLUS AND AFLATOXINS

Muhammad Kashif [1], Muhammad Riaz [2,*], Kanza Saeed[3], Amir Ismail [2], Muhammad Sameem Javed[2*], Mumtaz Hussain[2]

[1] Department of Food Science & Technology, Faculty of Food Science and Nutrition, Bahauddin Zakariya University, Multan, 60800 Pakistan
[2] Department of Food Safety & Quality Management, Faculty of Food Science and Nutrition, Bahauddin Zakariya University, Multan, 60800 Pakistan.
[3]Faculty of Food Technology and Nutrition Sciences, University of Biological and Applied Sciences, Lahore, Pakistan.
*Corresponding authors: Muhammad Riaz, riaz@bzu.edu.pk; Muhammad Sameem Javed, sameemjaved@bzu.edu.pk.

ABSTRACT

Aflatoxins, toxic metabolites produced mainly by the Aspergillus flavus (Af) and A. parasiticus (Ap), are major contaminants in food commodities including brown rice, posing severe health and economic challenges. This study investigated the inhibitory potential of aqueous extracts (AE) from four medicinal plants, Calotropis gigantea (C.g), Azadirachta indica (A.i), Eucalyptus globulus (E.g), and Spinacia oleracea (S.o) against the growth of Aspergillus flavus (Af) and Aspergillus parasiticus (Ap), as well as the aflatoxins production in Pakistani Super Kernel Basmati brown rice. A completely randomized design (CRD) was used for the experiment. The plant aqueous extracts (AE) were applied in 3 different concentrations (0.25, 0.5 and 0.75% w/v), and the antifungal and anti-aflatoxins effects were evaluated on the 0, 7th, and 14th days of storage. The inhibitory potential of all four medicinal plants' AE against Af and Ap and aflatoxins formation followed the order: A.i > E.g > C.g > S.o. The mean maximum antifungal activity against Af and Ap and aflatoxins inhibition potential was recorded at 0.75 % concentration of AE from A.i plant on the 0 day, while the mean minimum antifungal and aflatoxins inhibition potential was recorded at 0.25% concentration of AE from S.o on the 14th day. The study underscores the significant potential of plant-based extracts as eco-friendly bio-preservatives to mitigate aflatoxins contamination, ensuring food safety and reducing reliance on synthetic fungicides.

KEYWORDS: Aqueous plant extracts, Aspergillus flavus, Aspergillus parasiticus, aflatoxins, brown rice.

1. INTRODUCTION

Aflatoxins are toxic secondary metabolites produced predominantly by Aspergillus flavus (Af) and A. parasiticus (Ap), with rice being a particularly susceptible commodity (Karami-Osboo et al., 2020; Pazla et al., 2024). Although more than 20 aflatoxins have been identified, aflatoxins B_1, B_2, G_1, and G_2 are the most prevalent and toxic; their combined concentration in food is referred to as total aflatoxins (TAF) (Cui et al., 2025; Khashan et al., 2025; Ullah et al., 2025a). Rice is especially prone to fungus contamination due to high moisture content at harvest and agronomic practices like excessive nitrogen fertilization, improper field waste water management, dense planting, delayed harvesting, thereby increasing risks to human and animal health (Choudhary et al., 2010; Siruguri et al., n.d.). Pakistani Super Kernel Basmati rice, valued globally for its distinctive aroma, flavor, and texture, faces reduced market demand partly due to fungal contamination, alongside challenges such as climatic variability, trade routes, and transportation costs (Saeed et al., n.d.). To facilitate international trade and protect consumers, regulatory authorities have settled maximum limits for aflatoxins in rice. The European Union permits 2 µg/kg for AFB_1 and 4 µg/kg for TAF (EU, 2023), while the Punjab Food Authority allows up to 20 µg/kg TAF in foodstuffs, including rice (GOP, 2018). Despite extensive research, no economically viable physical, chemical, or biological method has been developed to effectively control fungal growth and aflatoxin production in food commodities, including rice (Gong et al., n.d.). Although several aflatoxin decontaminations approaches have been proposed, many compromises nutritional quality and generate toxic byproducts during aflatoxin biodegradation (Méndez-Albores et al., 2008; Sadimantara et al., 2024; Tabata et al., 1994). with Thus, there remains a critical need for the development of innovative, eco-friendly, non-toxic, and biodegradable strategies to mitigate fungal contamination and aflatoxin production (Naeem et al., 2026).

Medicinal plant extracts represent a promising strategy for controlling mold growth and aflatoxin production (Aamir et al., 2025; Aziz et al., 2025; Iqbal et al., 2024; Karabacak et al., 2025; Naeem et al., 2026; Rai et al., 2025; F. Tian et al., 2022). Over 53,000 medicinal plant species have been identified worldwide, many of which contain antifungal bioactive compounds, including phytoalexins, phenolics, thiosulfonates, and flavonoids (Bagheri et al., 2024), capable of suppressing fungal growth and aflatoxin biosynthesis (Ehab et al., 2025; Gul et al., 2024; Kumar et al., 2017; Rivera et al., 2025; Vila-Donat et al., 2018). In India, (Reddy et al., 2009) have reported that Syzygium aromaticum (0.5%) completely inhibited the growth of Af and AFB_1 production in rice, while Ocimum sanctum, Curcuma longa, and Allium sativum reduced Af growth (65–78%) and AFB_1 levels (72.2–85.7%) at the same (0.5%) concentration. The selection of Azadirachta indica, Eucalyptus globulus, Calotropis gigantea, and Spinacia oleracea as bio-preservatives is strategically supported by recent evidence regarding their multi-target antifungal mechanisms. Recent studies highlight that secondary metabolites such as azadirachtin, 1,8-cineole, and various cardiac glycosides act not only by disrupting fungal plasma membrane integrity but also by suppressing the enzymatic pathways responsible for the transition from primary to secondary metabolism in Aspergillus species. For instance, recent evaluations of botanical extracts have demonstrated significant inhibitory effects against aflatoxigenic fungi (Almusallam et al., 2025; Neira-Mosquera et al., 2025), with reductions in total aflatoxins (TAF) and AFB_1 exceeding 92.5% and 88.4% respectively, particularly when applied to high-carbohydrate matrices (Hussain et al., 2012; Ullah et al., 2025b). Despite these advancements, a significant knowledge gap exists regarding the efficacy of these aqueous extracts specifically within the matrix of Super Kernel Basmati brown rice. This variety distinct nutrient profile, characterized by high lipid and bran content, presents a unique biochemical environment for fungal colonization that is not addressed in standard solvent-based studies. By investigating cost-effective, "zero-residue" aqueous treatments against indigenous Pakistani strains of A. flavus and A. parasiticus, this research addresses the urgent need for sustainable technologies in the Pakistani export sector, which faces increasingly stringent international regulations on mycotoxin limits (Naeem et al., 2026). Evaluating these four plants in a comparative hierarchy ranging from potent medicinal flora to common dietary staples like S. oleracea fills a critical academic void by providing a novel framework for preserving premium rice commodities. Although the aqueous extract of Calotropis gigantea, Eucalyptus globulus, Azadirachta indica, and Spinacia oleracea has demonstrated strong antifungal activity in other systems, their efficacy in key Pakistani rice variety like Super Kernel Basmati brown rice remains unexplored, a gap that the current study addresses. Accordingly, the present study investigates the efficacy of aqueous extracts of these plants against Af and Ap growth and aflatoxin production, with the aim of informing a sustainable, natural preservation approach for rice to enhance food safety and quality.

2. MATERIALS AND METHODS

2.1. Chemicals and reagents

Aflatoxin standard solution (Aflatoxin-Mix, CRM46303), and trifluoroacetic acid were sourced from Romer Labs (Getzersdorf, Austria) and Sigma-Aldrich (St. Louis, Missouri, USA), respectively. Other chemicals, including KCl, $NaHCO_3$, NaCl, HPLC-grade acetonitrile and methanol were acquired from Merck (Darmstadt, Germany), while Aspergillus spp. differentiation medium base was acquired from Himedia (India).

2.2. Plant extracts preparation

Four medicinal plants, Calotropis gigantea (C.g), Eucalyptus globulus (E.g), Azadirachta indica (A.i), and Spinacia oleracea (S.o), were collected from Multan, Pakistan. However, C.g, E.g, and A.i were sourced from Bahauddin Zakariya University (BZU), while S.o leaves were obtained from local markets. In 2025, plant materials were authenticated by the Department of Botany, BZU, and processed at the Faculty of Food Science and Nutrition, BZU. Leaves were washed, oven-dried (50 °C), and ground to fine powder (100 µm). Powdered samples were extracted in distilled water (0.25, 0.5, and 0.75% m/v; 200 mL) with shaking at 280 rpm for 8 h at 25 °C, followed by incubation at dark for 12 h. Extracts were filtered through muslin cloth and Whatman No. 41 filter paper, concentrated using a rotary vacuum evaporator at 50 °C (Heidolph, Germany), and stored at 4-7 °C until analysis (Ponzilacqua et al., 2019).

2.3. Preparation of brown rice samples for fungal growth and aflatoxins production

2.3.1 Sterilization and preparation of Aspergillus inoculum

Brown rice samples (5 g) were placed in glass Petri dishes and independently sterilized at 121 °C for 15 min prior to spiking with the strains of Af (SRRC 1273) and Ap (SRRC 143) obtained from the Department of Plant Pathology, BZU, Pakistan. Fungal inocula were prepared separately on potato dextrose agar slants and incubated (25 °C/24 h). Following growth, spores were harvested with sterile distilled water and homogenized by vortexing for 2–3 min. Aqueous plant extracts (0.25, 0.5, and 0.75%) were applied to rice samples prior to inoculation with 1.0 mL of spore suspension adjusted to 10^{-5} by serial dilution. Treated samples were incubated at 25 °C for 14 days to assess the inhibitory effects of plant extracts on fungal growth and aflatoxin production (Madhyastha and Bhat, 1984).

2.3.2. Assessment of Af and Ap after treatment with aqueous extracts of medicinal plants

The method reported in a previous study (Phoku et al., 2016) was adopted to determine Af and Ap counts on the 0, 7th, and 14th days of storage with the help of a hemocytometer after thorough mixing. A Ringer's solution for serial dilution was prepared by adding 6.5 g NaCl, 0.25 g $CaCl_2$, 0.2 g $NaHCO_3$, and 0.42 g KCl into 1.0 L of sterilized distilled water, until achieving a 10^{-4} dilution. Then, 0.1 mL was taken from each dilution and poured onto a hemocytometer to count the spores of Af and Ap under microscope.

2.4. Aflatoxin analysis

TAF were analyzed in rice samples on days 0, 7, and 14 of storage. Briefly, extraction of TAF was performed using 100 mL of methanol:water (60:40, v/v) added to 25 g of infected rice samples. Mixtures were then incubated on a laboratory shaker (Thermo Scientific, Waltham, MA, USA) at a speed of 200 rpm for 4-5 h at 25 ˚C. Extracts were filtered (2.5 µm), and 4 mL of filtrate was diluted with 16 mL of 50 mM phosphate-buffered saline (PBS, pH 7.4). Purification was performed using immunoaffinity columns (Romer Labs, Pakistan) at a gravity flow rate of 1-3 mL/min. Columns were washed with 10 mL PBS:methanol (90:10, v/v), and aflatoxins were eluted with 2 mL methanol.

AFB$_1$ and AFG$_1$ were derivatized according to AOAC Official Method 994.08 method prior to TAF determination (AOAC, 2023). Dried extracts and standards were subjected to trifluoroacetic acid (50 µL) and hexane (200 µL) in sealed vials and incubated in the dark (5–6 min). Subsequently, 1.95 mL of distilled water:acetonitrile (1:9, v/v) was added, and samples were vortexed (1–2 min) using a vortex (VMX3-28, Infitek, China). The aqueous phase was collected and filtered through 0.45 µm syringe filters (Millex-HV, SLHV033R) for analysis. Quantification was performed using a high-performance liquid chromatographic (HPLC) system (Sykam S-500 routine series) with a mobile phase of methanol:water:acetonitrile (22.5:55:22.5, v/v/v) at a flow rate of 1.0 mL/min. The injection volume was 20 µL, total run time was 20 min, and column temperature was maintained at 37 °C. Detection was achieved using a fluorescence detector (excitation 365 nm; emission 440 nm). Retention times for AFG$_1$, AFB$_1$, AFG$_2$, and AFB$_2$ were 4.28, 5.09, 6.66, and 8.78 min, respectively.

The performance of the HPLC method for TAF quantification was evaluated in terms of linearity, limits of detection (LOD) and quantification (LOQ), and recovery. Calibration curves were constructed using mixed aflatoxin standards in acetonitrile at 3, 6, 9, and 12 µg/kg, yielding coefficients of determination (R^2) of 0.9984 for AFB$_1$, 0.9998 for AFG$_2$, 0.9992 for AFG$_1$, and 0.9989 for AFB$_2$. The LODs, determined following (Kortei et al., 2021) were 0.05 µg/kg for AFB$_1$ and AFG$_1$ and 0.03 µg/kg for AFB$_2$ and AFG$_2$, while the corresponding LOQs were 0.15 µg/kg and 0.09 µg/kg, respectively. Mean recovery rates ranged from 91–97% for AFB$_1$, 94–97% for AFG$_1$, 96–99% for AFB$_2$, and 96–98% for AFG$_2$.

3. STATISTICAL ANALYSIS

Statistical analysis was performed using Statplus software. Each sample was tested in triplicate to minimize errors and the results are expressed as mean± standard deviation. Microsoft Excel 2013 was used to perform data calculations. The experiment was arranged in a completely randomized design (CRD) with the two factors i.e., Plant type and extract concentration. Data was analyzed using two-way analysis of variance with post hoc Tukey's Test to evaluate the effect of factors and their interaction

4. RESULTS

4.1. ANTIFUNGAL potential of aqueous extracts of medicinal plants

The antifungal activity of aqueous extracts against Af and Ap in brown rice varied significantly with plant species, extract concentration, and storage duration, as summarized in Table 1. The inhibition was highest on day 0 and declined progressively by days 7 and 14 ($p < 0.05$), while the negative control showed no antifungal activity.

Table 1. Antifungal potential (expressed as % inhibition) of aqueous extracts (AE) of medicinal plant extracts applied on Aspergilli-inoculated brown rice.

Storage duration (days)	Plant species	AE concentration (%)	Inhibition of Af (%) ^{1}	Inhibition of Ap (%) ^{1}
0	-	0.00	0.00±0.00^{l}	0.00±0.00^{k}
	A. indica	0.25	92.25±14.01^{c}	93.62±11.55^{d}
		0.50	93.38±11.95^{b}	95.39±8.34^{b}
		0.75	95.13±8.81^{a}	97.15±5.17^{a}
	E. globulus	0.25	86.71±19.19^{f}	91.24±15.86^{f}
		0.50	90.09±15.53^{d}	92.86±12.91^{e}
		0.75	92.19±13.13^{c}	94.34±10.25^{c}
	C. gigantea	0.25	80.99±20.45^{i}	88.34±17.94^{h}
		0.50	85.31±17.85^{g}	91.35±14.99^{f}
		0.75	89.23±14.90^{e}	93.04±12.60^{e}
	S. oleracea	0.25	71.34±20.03^{k}	80.21±18.58^{j}
		0.50	75.52±18.10^{j}	83.28±18.04^{i}
		0.75	81.85±16.77^{h}	89.13±14.51^{g}
7th	-	0.00	0.00±0.00^{k}	0.00±0.00^{l}
	A. indica	0.25	88.91±20.06^{c}	91.06±16.17^{c}
		0.50	91.24±15.83^{b}	93.08±12.52^{b}
		0.75	92.68±13.23^{a}	94.54±9.88^{a}
	E. globulus	0.25	67.76±15.63^{f}	74.94±13.26^{gh}
		0.50	73.49±13.59^{e}	80.60±11.70^{f}
		0.75	81.96±12.06^{d}	89.54±10.32^{d}
	C. gigantea	0.25	58.05±17.78^{h}	65.97±15.97^{j}
		0.50	64.43±15.79^{g}	74.19±14.39^{h}
		0.75	73.87±12.96^{e}	83.18±10.32^{e}
	S. oleracea	0.25	48.87±19.27^{j}	58.59±18.36^{k}
		0.50	56.28±17.65^{i}	67.46±15.54^{i}
		0.75	65.13±15.53^{g}	75.41±14.49^{g}

14th	-	0.00	0.00±0.00^{i}	0.00±0.00^{k}
	A. indica	0.25	15.81±8.44^{c}	22.28±10.23^{c}
		0.50	22.78±8.00^{b}	29.11±8.33^{b}
		0.75	30.61±7.33^{a}	35.90±7.73^{a}
	E. globulus	0.25	7.27±2.59^{f}	17.51±11.0^{f}
		0.50	13.67±5.10^{d}	19.31±7.32^{e}
		0.75	22.36±5.71^{b}	28.74±8.46^{b}
	C. gigantea	0.25	3.58±1.62^{g}	7.89±3.27^{i}
		0.50	7.51±2.53^{f}	12.07±3.92^{h}
		0.75	14.08±6.91^{d}	20.15±8.84^{d}
	S. oleracea	0.25	1.47±0.86^{h}	4.28±2.16^{j}
		0.50	3.39±1.31^{g}	8.02±2.56^{i}
		0.75	8.35±5.69^{e}	14.27±7.17^{g}

1 Values expressed as ± standard deviation of samples analyzed in triplicate.
a-1 means followed by different superscript letters within the same columns and day of storage group differ significantly (P < 0.05). AE: aqueous extract; Af: Aspergillus flavus; Ap: Aspergillus parasiticus.

Among the tested plants, A. indica exhibited the strongest antifungal effect at all concentrations, with 0.75 % extract achieving the maximum inhibition against Ap and Af on day 0 (97.15 ± 5.17% and 95.13 ± 8.81%, respectively), day 7 (94.54 ± 9.88% and 92.68 ± 13.23%), and day 14 (35.90 ± 7.73% and 30.61 ± 7.33%). In contrast, S. oleracea showed the weakest activity, particularly at 0.25%, with inhibition declining to 4.28 ± 2.16% for Ap and 1.47 ± 0.86% for Af by day 14. Intermediate antifungal efficacy was observed for E. globulus and C. gigantea. Notably, the antifungal potential of the evaluated medicinal plants followed the order A. indica > E. globulus > C. gigantea > S. oleracea (Table 1).

On Day 0, two-way ANOVA demonstrated that both plant type and treatment concentration had a statistically significant effect ($p < 0.05$) on the inhibition of Aspergillus flavus (Af) and A. parasiticus (Ap). For Af inhibition, the plant factor was significant ($p = 0.0069$), indicating differences in antifungal activity among the tested extracts. Tukey's HSD analysis revealed that Azadirachta indica (Neem) showed significantly greater inhibition compared to Spinacia oleracea (Spinach) ($p < 0.05$), while no significant differences were observed between Neem and Eucalyptus globulus (Sufaida) or Calotropis gigantea (Auk). Additionally, Spinach differed significantly from Sufaida ($p < 0.05$), whereas other comparisons were not significant.

Similarly, for Ap inhibition, the plant effect was significant ($p = 0.0088$). Post-hoc analysis showed that Neem exhibited significantly higher inhibition than Spinach ($p < 0.05$), while differences among the remaining plant pairs were not statistically significant, except between Spinach and Sufaida, which also showed a significant difference ($p < 0.05$).

The treatment factor showed a highly significant effect ($p < 0.001$) for both Af and Ap inhibition, indicating a strong reduction in fungal growth due to extract application. Pairwise comparisons confirmed that all treatment groups (T1, T2, and T3) differed significantly from the control (T0), whereas no significant differences were observed among the treatment levels themselves ($p > 0.05$). Overall, the results indicate that both plant type and treatment significantly influence antifungal activity on Day 1, with the primary variation arising from differences between treated and untreated samples and comparatively higher inhibition associated with Neem relative to Spinach.

On Day 7, two-way ANOVA indicated that both plant type and treatment concentration had a statistically significant effect ($p < 0.05$) on the inhibition of Aspergillus flavus (Af) and A. parasiticus (Ap). For Af inhibition, the plant factor was significant ($p = 0.0064$), demonstrating variation in antifungal efficacy among the tested extracts. Tukey's HSD analysis showed that Azadirachta indica (Neem) exhibited significantly greater inhibition compared to Calotropis gigantea (Auk) and Spinacia oleracea (Spinach) ($p < 0.05$), while differences between Neem and Eucalyptus globulus (Sufaida), as well as among the remaining plant comparisons, were not statistically significant.

Similarly, for Ap inhibition, the plant effect was significant ($p = 0.0089$). Post-hoc comparisons confirmed that Neem showed significantly higher inhibition than Auk and Spinach ($p < 0.05$), whereas no significant differences were observed between Neem and Sufaida or among the other plant pairs.

The treatment factor showed a highly significant effect ($p < 0.001$) for both Af and Ap inhibition, indicating a substantial reduction in fungal growth due to extract application. Pairwise comparisons revealed that all treatment groups (T1, T2, and T3) differed significantly from the control (T0), while no significant differences were observed among the treatment concentrations themselves ($p > 0.05$). Overall, the results on Day 7 demonstrate that both plant type and treatment significantly influence antifungal activity, with Neem showing comparatively higher inhibitory effects than some plant extracts and the primary differences driven by treated versus untreated samples.

On Day 14, two-way ANOVA showed that both plant type and treatment concentration had a statistically significant effect ($p < 0.05$) on the inhibition of Aspergillus flavus (Af) and A. parasiticus (Ap). For Af inhibition, the plant factor was significant ($p = 0.0072$), indicating differences in antifungal efficacy among the tested extracts. Tukey's HSD analysis revealed that Azadirachta indica (Neem) exhibited significantly greater inhibition compared to Calotropis gigantea (Auk) and Spinacia oleracea (Spinach) ($p < 0.05$), while differences between Neem and Eucalyptus globulus (Sufaida), as well as among other plant comparisons, were not statistically significant.

Similarly, for Ap inhibition, the plant effect was significant ($p = 0.0052$). Post-hoc comparisons confirmed that Neem showed significantly higher inhibition than Auk and Spinach ($p < 0.05$), whereas differences between Neem and Sufaida and among the remaining plant pairs were not statistically significant.

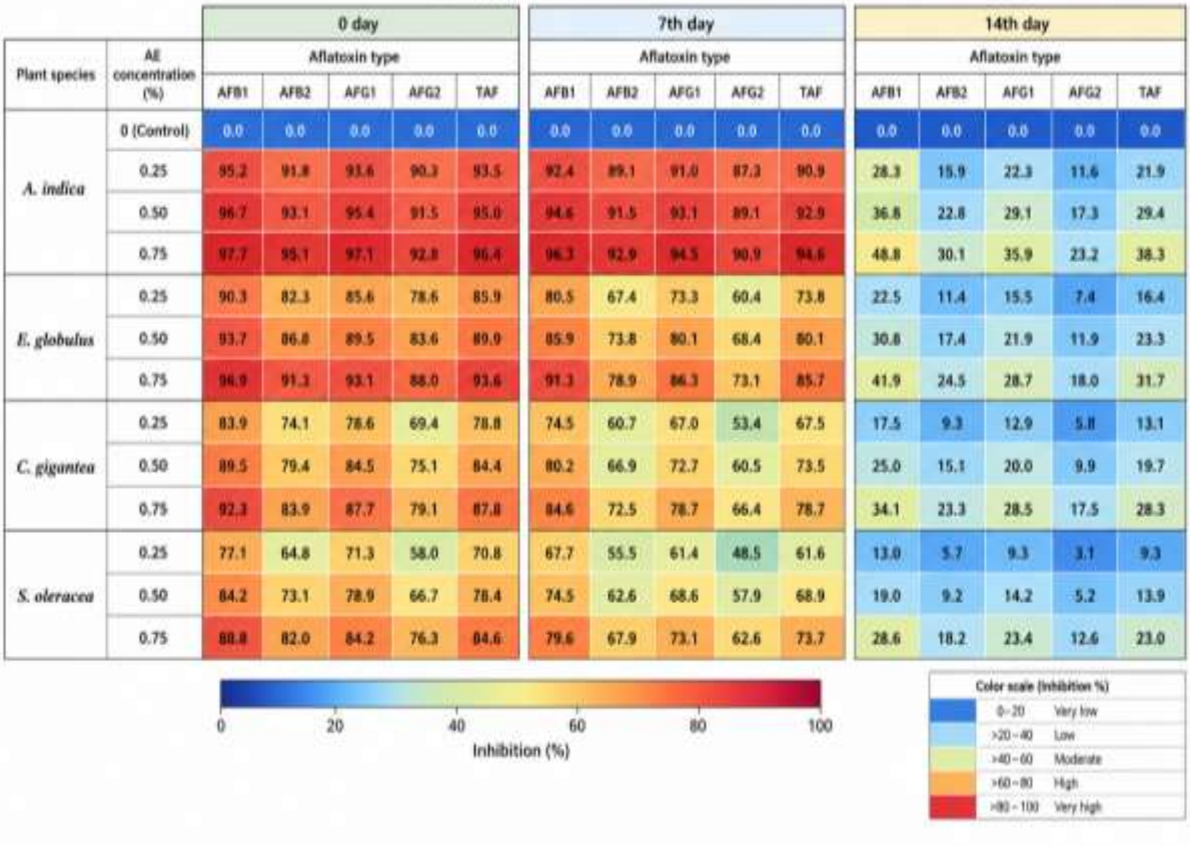
The treatment factor also showed a significant effect for both Af ($p = 0.0012$) and Ap ($p = 0.00024$), indicating that extract application reduced fungal growth. For Af inhibition, significant differences were observed between the control (T0) and higher treatment levels (T2 and T3), while the comparison between T0 and T1 was not significant. Additionally, T3 showed significantly greater inhibition compared to T1 ($p < 0.05$). For Ap inhibition, all treatment groups (T1, T2, and T3) differed significantly from the control (T0), and T3 also showed significantly greater inhibition compared to T1 ($p < 0.05$), while other comparisons among treatment levels were not significant.

Overall, the results on Day 14 indicate that both plant type and treatment concentration significantly influence antifungal activity, with Azadirachta indica consistently demonstrating higher inhibitory effects compared to certain plant extracts, and a more pronounced concentration-dependent response observed at this later stage of storage.

4.2. Aflatoxin inhibition potential of selected medicinal plant extracts

The aflatoxin inhibition potential of selected indigenous medicinal plant extracts at varying concentrations is presented in Figure 1. All extracts significantly inhibited AFB_1, AFB_2, AFG_1, AFG_2, and TAF in a concentration- and storage time-dependent manner ($p < 0.05$). Inhibition was highest on day 0 and declined significantly by days 7 and 14, while untreated controls showed no inhibition.

Figure 1: Aflatoxin Inhibition (%) Of Plant Extarct With Varying Concentrations At Different Storage Periods



Plant species	AE concentration (%)	0 day					7th day					14th day				
		Aflatoxin type					Aflatoxin type					Aflatoxin type				
		AFB1	AFB2	AFG1	AFG2	TAF	AFB1	AFB2	AFG1	AFG2	TAF	AFB1	AFB2	AFG1	AFG2	TAF
A. indica	0 (Control)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	0.25	95.2	91.8	93.6	90.3	93.5	92.4	89.1	91.0	87.3	90.9	28.3	15.9	22.3	11.6	21.9
	0.50	96.7	93.1	95.4	91.5	95.0	94.6	91.5	93.1	89.1	92.9	36.8	22.8	29.1	17.3	29.4
	0.75	97.7	95.1	97.1	92.8	96.4	96.3	92.9	94.5	90.9	94.6	48.8	30.1	35.9	23.2	38.3
E. globulus	0.25	90.3	82.3	85.6	78.6	85.9	80.5	67.4	73.3	60.4	73.8	22.5	11.4	15.5	7.4	16.4
	0.50	93.7	86.8	89.5	83.6	89.9	85.9	73.8	80.1	68.4	80.1	30.8	17.4	21.9	11.9	23.3
	0.75	96.9	91.3	93.1	88.0	93.6	91.3	78.9	86.3	73.1	85.7	41.9	24.5	28.7	18.0	31.7
C. gigantea	0.25	83.9	74.1	78.6	69.4	78.8	74.5	60.7	67.0	53.4	67.5	17.5	9.3	12.9	5.8	13.1
	0.50	89.5	79.4	84.5	75.1	84.4	80.2	66.9	72.7	60.5	73.5	25.0	15.1	20.0	9.9	19.7
	0.75	92.3	83.9	87.7	79.1	87.8	84.6	72.5	78.7	66.4	78.7	34.1	23.3	28.5	17.5	28.3
S. oleracea	0.25	77.1	64.8	71.3	58.0	70.8	67.7	55.5	61.4	48.5	61.6	13.0	5.7	9.3	3.1	9.3
	0.50	84.2	73.1	78.9	66.7	78.4	74.5	62.6	68.6	57.9	68.9	19.0	9.2	14.2	5.2	13.9
	0.75	88.8	82.0	84.2	76.3	84.6	79.6	67.9	73.1	62.6	73.7	28.6	18.2	23.4	12.6	23.0

Color scale (Inhibition %)		
🟦	0–20	Very low
🟦	>20–40	Low
🟨	>40–60	Moderate
🟧	>60–80	High
🟥	>80–100	Very high

Among the tested plants, A. indica exhibited the strongest anti-aflatoxin activity, particularly at 0.75 %. On day 0, A. indica (0.75 %) reduced AFB_1, AFB_2, AFG_1, AFG_2, and TAF by 97.7 ± 4.19%, 95.1 ± 8.93%, 97.1 ± 5.17%, 92.8 ± 12.9%, and 96.4 ± 6.43%, respectively. Corresponding inhibition on day 7 remained high (AFB_1: 96.3 ± 6.62%; TAF: 94.6 ± 9.73%) but declined by day 14 (AFB_1: 48.8 ± 10.9%; TAF: 38.3 ± 8.91%). In contrast, S. oleracea showed the weakest inhibition, particularly at 0.25%, with reductions on day 0 of 77.1 ± 13.0% (AFB_1), 64.8 ± 12.2% (AFB_2), 71.3 ± 12.3% (AFG_1), 58.0 ± 12.0% (AFG_2), and 70.8 ± 12.5% (TAF), decreasing sharply by day 14 to 13.0 ± 9.76% (AFB_1) and 9.26 ± 7.41% (TAF). E. globulus and C. gigantea exhibited moderate inhibition across all aflatoxins and storage periods (Figure 1). Generally, the inhibition percentages were significantly higher on day 0 than that observed on days 7 and 14 ($P < 0.05$). The overall efficacy of the tested medicinal plants in suppressing aflatoxin production followed the order A. indica > E. globulus > C. gigantea > S. oleracea (Figure 1).

On Day 0, the two-way ANOVA demonstrated that both plant type and treatment concentration had a statistically significant effect ($p < 0.05$) on all measured aflatoxin parameters, including AFB1, AFB2, AFG1, AFG2, and total aflatoxins (TAF). The effect of plant extracts was significant across all responses, indicating variability in antifungal efficacy among the tested species. Tukey's HSD post-hoc analysis revealed that Azadirachta indica (Neem) exhibited significantly greater inhibition of aflatoxin production compared to Spinacia oleracea (Spinach) ($p < 0.05$), whereas differences between Neem and Eucalyptus globulus (Sufaida), as well as between Calotropis gigantea (Auk) and the other plant extracts, were not statistically significant.

The treatment factor showed a highly significant effect ($p < 0.001$) for all aflatoxin types, indicating that the application of plant extracts markedly reduced aflatoxin levels compared to the control. Pairwise comparisons confirmed that all treatment levels (T1, T2, and T3) differed significantly from the control group (T0). However, no significant differences were observed among the treatment concentrations themselves (T1 vs T2, T1 vs T3, and T2 vs T3; $p > 0.05$), suggesting that increasing concentration did not produce statistically distinct effects on Day 1. Overall, the results indicate that both the type of plant extract and its application significantly influence aflatoxin inhibition, with the primary variation arising from differences between treated and untreated samples.

On Day 7, two-way ANOVA indicated that both plant type and treatment concentration had a statistically significant effect ($p < 0.05$) on all aflatoxin parameters, including AFB1, AFB2, AFG1, AFG2, and total aflatoxins (TAF). The plant factor showed significant variation across all responses (p-values ranging from 0.005 to 0.006), confirming differences in inhibitory potential among the tested extracts. Tukey's HSD analysis demonstrated that Azadirachta indica (Neem) exhibited significantly greater inhibition compared to Calotropis gigantea (Auk) and Spinacia oleracea (Spinach) across all aflatoxin types ($p < 0.05$). However, differences between Neem and Eucalyptus globulus (Sufaida), as well as among the remaining plant comparisons, were not statistically significant ($p > 0.05$).

The treatment factor exerted a highly significant effect ($p < 0.001$) on all responses, indicating a strong reduction in aflatoxin levels due to extract application. Post-hoc comparisons revealed that all treatment groups (T1, T2, and T3) differed significantly from the control (T0), whereas no significant differences were observed among the treatment levels themselves ($p > 0.05$). Overall, the results on Day 7 confirm that both plant type and treatment significantly influence aflatoxin inhibition, with the primary differences driven by the superiority of Neem over certain plants and the clear distinction between treated and untreated samples.

On Day 14, two-way ANOVA revealed that both plant type and treatment concentration continued to have a statistically significant effect ($p < 0.05$) on all aflatoxin parameters, including AFB1, AFB2, AFG1, AFG2, and total aflatoxins (TAF). The plant factor showed consistent significance across all responses ($p \approx 0.005–0.006$), indicating persistent differences in inhibitory potential among the tested extracts. Tukey's post-hoc analysis demonstrated that Azadirachta indica (Neem) maintained significantly greater inhibition compared to Calotropis gigantea (Auk) and Spinacia oleracea (Spinach) for most aflatoxin types ($p < 0.05$), while differences between Neem and Eucalyptus globulus (Sufaida), as well as among other plant comparisons, were generally not statistically significant.

The treatment factor also showed a highly significant effect ($p \leq 0.00001$), confirming that extract application substantially reduced aflatoxin levels compared to the control. Pairwise comparisons indicated that all treatment levels (T1, T2, and T3) differed significantly from the control (T0). Unlike earlier storage days, several significant differences were observed among treatment concentrations on Day 14. In particular, higher concentrations (T3) showed significantly greater inhibition compared to lower concentrations (T1 and, in some cases, T2), especially for AFB1, AFB2, AFG1, and TAF. This suggests a more pronounced concentration-dependent effect at prolonged storage duration.

Overall, the results on Day 14 indicate that both plant type and concentration remain critical factors influencing aflatoxin inhibition, with Azadirachta indica showing relatively stronger and more consistent inhibitory effects, and treatment differences becoming more distinct over time.

Pearson's correlation Analysis

Pearson's correlation analysis demonstrated a highly significant ($p < 0.001$) and very strong positive relationship among all measured aflatoxins (AFB1, AFB2, AFG1, AFG2) and total aflatoxins (TAF), with correlation coefficients ranging from 0.969 to 0.999. This indicates that the different aflatoxin types are closely associated and likely produced simultaneously under similar conditions, reflecting a common biosynthetic origin and shared fungal growth behavior. Total aflatoxins (TAF) showed an almost perfect correlation with individual aflatoxins, which is expected since it represents their combined concentration. In addition, both aflatoxin inhibition and fungal inhibition exhibited strong positive correlations with individual and total aflatoxins, with coefficients generally exceeding 0.97. Notably, the correlation between fungal inhibition and aflatoxin inhibition was also extremely high ($r = 0.994$), suggesting that reductions in fungal growth are strongly associated with decreased aflatoxin production. This supports the hypothesis that the tested medicinal plants exert their effect primarily by inhibiting fungal proliferation, thereby limiting toxin synthesis. However, the exceptionally high correlation values may also indicate interdependence among variables, particularly if total aflatoxins and inhibition percentages were derived from the same underlying data, and thus should be interpreted with caution when inferring causality.

Table 2: Pearson's Correlation Matrix Among Aflatoxxins, Total Aflatoxins and Inhibition Parameters

R	AFB1	AFB2	AFG1	AFG2	TAF	Inhibition Af	Inhibition p
AFB1	1						
p-value (2-tailed)							
AFB2	0.98553	1					
p-value (2-tailed)	0						
AFG1	0.99463	0.9967	1				
p-value (2-tailed)	0	0					
AFG2	0.96962	0.99638	0.98748	1			
p-value (2-tailed)	0	0	0				
TAF	0.99607	0.99653	0.99961	0.98705	1		
p-value (2-tailed)	0	0	0	0			
Inhibition Af	0.97057	0.98624	0.98258	0.98537	0.98245	1	
p-value (2-tailed)	0	0	0	0	0		
Inhibition p	0.98346	0.98566	0.98827	0.97798	0.98851	0.99408	1
p-value (2-tailed)	0	0	0	0	0	0	

Correlations in bold are significant at the 5% level (2-tailed).
N of valid cases = 576.

Table 3: Pairwise correlation Coefficients (R Value) for aflatoxins and Inhibition Parameters

VAR vs. VAR	R	N	p-value
TAF vs. AFG1	0.99961	576	0
AFG1 vs. AFB2	0.9967	576	0
TAF vs. AFB2	0.99653	576	0
AFG2 vs. AFB2	0.99638	576	0
TAF vs. AFB1	0.99607	576	0
AFG1 vs. AFB1	0.99463	576	0
Inhibition p vs. Inhibition Af	0.99408	576	0
Inhibition p vs. TAF	0.98851	576	0
Inhibition p vs. AFG1	0.98827	576	0
AFG2 vs. AFG1	0.98748	576	0
TAF vs. AFG2	0.98705	576	0
Inhibition Af vs. AFB2	0.98624	576	0
Inhibition p vs. AFB2	0.98566	576	0
AFB2 vs. AFB1	0.98553	576	0
Inhibition Af vs. AFG2	0.98537	576	0
Inhibition p vs. AFB1	0.98346	576	0
Inhibition Af vs. AFG1	0.98258	576	0
Inhibition Af vs. TAF	0.98245	576	0
Inhibition p vs. AFG2	0.97798	576	0
Inhibition Af vs. AFB1	0.97057	576	0
AFG2 vs. AFB1	0.96962	576	0

5. DISCUSSION

A time-related decline in inhibition in this study is consistent with reduced availability and/or stability of bioactive constituents over storage (e.g., oxidation, volatilization, sorption into the food matrix), which lowers the effective concentration at the fungal interface (Leiva-Mora et al., 2025; Li et al., 2025). This strong early inhibition is mechanistically consistent with the rapid and multi-site antifungal actions (membrane permeability disruption and energy-metabolism impairment), which typically manifest rapidly after exposure and are frequently concentration dependent (Leiva-Mora et al., 2025; J. Tian et al., 2012). Such variations in efficacy across plant species are mechanistically attributable to differences in phytochemical composition and abundance (e.g., terpenoids, phenolics, aldehydes), which determine the strength of membrane, mitochondrial, and oxidative-stress effects in Aspergillus (Leiva-Mora et al., 2025; Li et al., 2025).

Plant extracts represent cost-effective, biodegradable and environmentally friendly alternatives to synthetic fungicides for mitigating fungal growth and aflatoxin contamination. Among over 400,000 compounds identified from medicinal plants, 10,000 secondary metabolites are considered natural defense compounds (Hamburger and Hostettmann, 1991; Phupaboon et al., 2025). In the current study four medicinal plant aqueous extracts exhibited marked difference in anti-fungal and anti-aflatoxigenic efficacy, following the order A. indica>E. globulus> C. giganten> S. oleracea. These variations can be mechanistically explained by differences in their phytochemical composition. The strong and sustained activity of A. indica extract achieved up to 97% inhibition on day 0 and maintained 90% inhibition through day 7, suggesting rich and stable profile of bioactive terpenoids like azadirachtin and nimbin. These bioactive phenols disrupt the integrity of plasma membrane, impair cell wall biosynthesis, and induce mitochondrial dysfunction (Leiva-Mora et al., 2025; J. Tian et al., 2012). In contrast to it the relatively weak potential of S. oleracea extracts with an inhibition dropping of 1..47% for Af and 428% for Ap by 14th dayindicates the rapid degradation of less potent flavonoids.

In line, studies worldwide have reported the antifungal potential of medicinal plants that support the present findings (Table 1). In Pakistan, (Hussain et al., 2012) evaluated several botanicals and reported inhibition trends closely corresponding with the present study. Similar findings were reported in Iraq (Al-Warshan et al., 2023), Bangladesh (Kuri et al., 2010), India (Reddy et al., 2009; Venkateswarlu et al., n.d.), and Indonesia (Sjam et al., 2018). Differences across studies are plausibly attributable to extract concentration, fungal strain, extraction method, and incubation duration (Gowda et al., 2004; Mohammedi et al., 2013). Strain- and inoculum-related modifications are mechanistically expected because membrane composition, oxidative-stress tolerance, and secondary metabolism regulation differ across isolates, changing susceptibility to phytochemical stress (Fountain, Bajaj, Nayak, et al., 2016; J. Tian et al., 2012). The antifungal effects of medicinal plants may be attributed to bioactive compounds present in A. indica, E. globulus, C. gigantea, and S. oleracea, which disrupt fungal membranes, alter membrane proteins, induce proton efflux, and destabilize cellular integrity (Leiva-Mora et al., 2025; Omidbeygi et al., 2007; J. Tian et al., 2012).

Plant extracts can suppress aflatoxin accumulation primarily by inhibiting aflatoxin biosynthesis, including downregulation of aflatoxin gene-cluster regulators (AflR and AflS) and structural biosynthetic genes, and by perturbing oxidative-stress signaling coupled to aflatoxin production in Aspergillus (Abbas et al., 2024; Caceres et al., 2020; Fountain, Bajaj, Pandey, et al., 2016; Sun et al., 2016). High early suppression is consistent with rapid transcriptional and stress-response effects that reduce aflatoxin pathway flux; the later decline can reflect reduced bioactive availability and/or fungal physiological adaptation after transient exposure (Leiva-Mora et al., 2025; Niu et al., 2023; Wang et al., 2019). Comparative studies report similar concentration- and time-dependent trends, including (Abeer et al., 2012; Al-Warshan et al., 2023; Gowda et al., 2004; Microbiology & 1997, 1997; Reddy et al., 2009; Vijayanandraj et al., 2014). Across studies, differences in inoculum, incubation time, and matrix conditions influence oxidative balance and AflR/AflS-regulated expression, thereby altering aflatoxin suppression even at similar extract concentrations (Abbas et al., 2024; Caceres et al., 2020; Fountain, Bajaj, Pandey, et al., 2016).

The variability in antifungal and anti-aflatoxigenic activity among medicinal plants may also reflect differences in phenolics, tannins, flavonoids, and pigments that neutralize aflatoxins through electron donation, structural modification, and complex formation (Dantas et al., 2025; Loi et al., 2020). In addition, chlorophyll and chlorophyllin can reduce aflatoxin bioavailability via complex formation that decreases absorption, supporting binding-based mitigation mechanisms (Breinholt et al., 1995; Jubert et al., 2009).

6. CONCLUSION

This study confirms the antifungal and anti-aflatoxigenic potential of aqueous extracts of Calotropis gigantea, Azadirachta indica, Eucalyptus globulus, and Spinacia oleracea against Aspergillus flavus (Af) and Aspergillus parasiticus (Ap) in Paksitani Super Kernel Basmati brown rice. Among all tested plants, A. indica at a concentration of 1% exhibited the strongest inhibition of fungal growth up to 97.7%, followed by , E. globulus> C. giganten> S. oleracea. Inhibition was highest on day 0 and progressively declined progressively by 7 and 14th day ($p<0.05$), with zero activity for control. The outcomes of the study demonstrate that A. indica aqueous extract is a promising, eco-friendly alternative to synthetic fungicide for short term protection of brown rice against aflatoxin contamination during storage.

CRediT authorship contribution statement

Muhammad Kashif: Methodology, Formal analysis, Writing - original draft. **Muhammad Riaz**: Investigation, Supervision, Writing–review & editing. **Kanza Saeed:** Data curation, Writing–review & editing. **Amir Ismail**: Conceptualization, Funding acquisition, Writing–review & editing. **Muhammad Sameem Javed**: Data curation, Writing–review & editing. **Mumtaz Hussain**: Visualization, Validation, Writing–review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability
Data will be made available on request.

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