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Inhibition of aflatoxin B1 biosynthesis and down regulation of *aflR* and *aflB* genes in presence of benzimidazole derivatives without impairing the growth of *Aspergillus flavus*

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ABSTRACT

Aflatoxins are mutagenic secondary metabolites produced by certain ubiquitous saprophytic fungi. These contaminate agricultural crops and pose a serious health threat to humans and livestock all over the world. Benzimidazole and its derivatives are biologically active heterocyclic compounds known for their fungicidal activity. In the present study, second and sixth position substituted benzimidazole derivatives are tested for their antifungal and anti-aflatoxigenic activity. Aflatoxigenic strain of *Aspergillus flavus* cultured in Yeast extract sucrose (YES) medium as well as in rice in the presence and absence of test compounds. 2-(2-Furyl) benzimidazole (FBD) showed complete inhibition of fungal growth at 50 μg/mL. However, the polar derivatives of FBD viz. 6-NFBD, 6-AFBD, 6-CAFBD, and 6-CFBD did not impair the fungal growth but effectively inhibited aflatoxin B_1 biosynthesis. Significant down-regulation of *aflR* gene involved in regulation and *aflB* structural gene for aflatoxin B_1 biosynthesis was observed in presence of 6-NFBD. These benzimidazole derivatives also showed good anti-aflatoxigenic activity in rice, though the IC_{50} concentrations in rice were comparatively higher than those in YES medium. This study summarizes the most notable structure-activity relationship (SAR) of 2-(2-Furyl) benzimidazoles for anti-aflatoxigenic and anti-fungal activities. These molecules can be further studied for their applications in industrial fermentation processes vulnerable to mold growth and subsequent aflatoxin B_1 synthesis like koji fermentation, cheese production, etc.

1. Introduction

Aflatoxins are a group of polyketide mycotoxins produced as secondary metabolites during fungal development (Herrman et al., 2002; Erdogan, 2004). Since aflatoxins directly influence the DNA structure, they are highly carcinogenic. Biosynthesis of aflatoxins by fungi depends on the environmental conditions such as moisture, temperature, and humidity during crop maturation and storage (Neme and Mohammed, 2017; Pildain et al., 2004; Tarín et al., 2004). Contamination of food, feed and agricultural commodities by aflatoxins impose an enormous economic burden (Mitchell et al., 2016) and the occurrence of aflatoxin in cereals causes trade issues between countries. Aflatoxin B_1 (AFB_1) contamination has been reported across the globe in various agricultural and dairy products. In Africa, about 50% of raw cereal grains have AFB_1 with highest incidence of 1642 μg/kg in rice and maize. In Asia and South America, these are reported to be 850 μg/kg and 1400 μg/kg in maize respectively (Lee and Ryu, 2017; Ferre, 2016). Europe has been recorded the highest contamination level of 656 μg/kg in food grains (Binder et al., 2007).

Good agricultural and harvesting practices and storage in dry conditions are helpful in preventing fungal infestation and thus mycotoxin synthesis (Magan and Aldred, 2007). These were expensive practices (Alaniz Zanon et al., 2013), and AFB_1 contamination remains a massive issue in developing countries such as Southeast Asia and India (Toteja et al., 2006). Alternatively, there are a number of physical and chemical methods such as heating, ionizing radiations, spraying of pesticides and fungicides for preventing fungal infestation and AFB_1 contamination. However, these methods can cause organoleptic changes to the product and irreversible damage to the environment due to their non-biodegradable nature (Divakara et al., 2014; Karlovsky et al., 2016; Zoghi et al., 2014). Thus the development of new methods for controlling aflatoxigenic fungi by naturally occurring spices (Aiko and Mehta, 2013a, 2013b) including plant extracts (Panda and Mehta, 2013) and plant compounds such as essential oils (Pandey et al., 2016) has been

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proposed as alternative fungicides to reduce AFB_1 (Hua et al., 2015; Kedia et al., 2014; Wright et al., 2000). Piperlongumine, pipernonaline, 2-methylenedioxy-containing compounds identified from *Piper nigrum*, piperonal and piperine have been studied for their antifungal and antiaflatoxigenic activities against *A. flavus*. 2-hydroxy-4-methoxybenzaldehyde isolated from *Decalepis hamiltonii* which exhibits antiaflatoxigenic activity (Mohana and Raveesha, 2010). The coumarin derivative, 7-hydroxy-6-nitro-2H-1-benzopyran-2-one, and benzimidazole derivatives are widely used because of their diverse biological activity and clinical application. This benzimidazole heterocyclic ring system presents fungicidal property (Janeczko et al., 2016; Guerra et al., 2015). 2-(2-Furyl)benzimidazole (FBD) commercially marketed as 'Fuberidazole' is well known for its fungicidal activity and commonly used against candida infection (Del Poeta et al., 1998). In quest of searching more potent fungicides, a number of more polar derivatives of FBD were synthesized and screened for their fungicidal activity. In the present work, the antifungal and anti-aflatoxigenic effects of benzimidazole containing compounds 2-(2-Furyl)-6-nitrobenzimidazole (6-NFBD), 2-(2-Furyl)benzimidazole-6-carbonitrile (6-CFBD), 2-(2-Furyl) benzimidazole-6-carboxamidoxime (6-AFBD), and 2-(2-Furyl)benzimidazole-6-carboxylic acid (6-CAFBD) were studied on *Aspergillus flavus*. Studies were carried out in culture media as well as in rice to establish the efficacy of these compounds in controlling AFB_1 contamination. The cytotoxicity to the HeLa cells was used as the assay for evaluation of the safety of the test compounds; FBD, 6-NFBD, 6-CFBD, 6-AFBD, 6-CAFBD.

2. Material and methods

2.1. General chemical synthesis

Most of the reagents and chemicals were obtained from Alfa Aesar (Ward Hill, MA), and Sigma-Aldrich (Milwaukee, WI). Benzimidazoles were synthesized using reported methods (Bahrami et al., 2007; Hein et al., 1957). The modified benzimidazoles synthesized at BogaR Laboratories for this study include 2-(2-Furyl)benzimidazole (FBD), 2-(2-Furyl)-6-nitrobenzimidazole (6-NFBD), 2-(2-Furyl)benzimidazole-6-carbonitrile (6-CFBD), 2-(2-Furyl)benzimidazole-6-carboxamidoxime (6-AFBD), and 2-(2-Furyl)benzimidazole-6-carboxylic acid (6-CAFBD) as shown in Fig. 1. Generally, all benzimidazole derivatives were prepared by refluxing the respective 1,2-phenylenediamine derivatives with 2-furfuraldehyde in nitrobenzene. The derived crude materials were crystallized from ethanol or a suitable solvent to give pure 2-(2-Furyl)benzimidazoles. All synthesized compounds were characterized by TLC, IR, and NMR. The purity of each compound was determined by TLC and NMR. The compounds used for our studies were well over 97% purity range.

2.1.1. 2-(2-Furyl) benzimidazole (FBD)

1,2-phenylenediamine (0.1 mol) was mixed with Furfural (Furan-2-carboxaldehyde) (0.12 mol) and nitrobenzene and then refluxed for 10 h. The reaction mixture was cooled and then poured into water. The precipitated solid was filtered and washed with water and dried to derive crude benzimidazole derivative, which crystallized from ethyl acetate-chloroform (1:1) to give crystalline 2-(2-Furyl)benzimidazole and yield was 80%, m.p. 200 °C (dec), ^{1}H-NMR (DMSO-d_6): δ 6.6–8.0 (aromatic protons, 7H), and 13.0 (imidazole amine-NH, 1H).

2.1.2. 2-(2-Furyl)-6-nitrobenzimidazole (6-NFBD)

Synthesized as described for FBD, except that 3,4-diaminonitrobenzene was used in place of the 1,2-phenylenediamine and yield was ~80%, m. p. 195–200 °C, ^{1}H-NMR (DMSO-d_6): δ 6.8–8.5 (aromatic protons, 6H), and 13.6 (broad singlet for imidazole-NH, 1H).

2.1.3. 2-(2-Furyl)benzimidazole-6-carbonitrile (6-CFBD)

6-CFBD was Synthesized as described for FBD, except that 3,4-diaminobenzonitrile was used in place of 1,2-phenylenediamine and the yield was ~90%, m.p. 149–152 °C, ^{1}H-NMR (DMSO-d_6): δ 6.6–8.2 (aromatic protons, 6H), and 13.6 (broad singlet for imidazole-NH, 1H).

2.1.4. 2-(2-Furyl)benzimidazole-6-carboxamidoxime (6-AFBD)

The above-synthesized compound (6-CFBD) was dissolved in 1,4-dioxane and then mixed with hydroxylamine solution and the reaction mixture was stirred for 12 h. After completion of the reaction, the solvent was evaporated under vacuum to dryness and then the derived solid was crystallized. Crystalline product yield was ~70% and m.p. 215–220 °C, ^{1}H-NMR (DMSO-d_6): δ 5.8 (amidoxime amine protons, 2H), 6.7–8.0 (aromatic protons, 6H), 9.5 (amidoxime hydroxyl proton, 1H), and 13.0 (imidazole-NH, 1H).

2.1.5. 2-(2-Furyl)benzimidazole-6-carboxylic acid (6-CAFBD)

6-CAFBD was synthesized as described for FBD, except that 3,4-diaminobenzoic acid was used in place of 1,2-phenylenediamine and the yield was ~80%, m.p. 200–204 °C, IR (KBr) spectrum showed a peak at 1680 cm^{-1} for Carbonyl group.

2.2. A. flavus culture

A toxigenic strain of *A. flavus* was isolated in our laboratory and identified as *Aspergillus flavus* link 1809 on the basis of partial DNA sequence similarity with NCBI sequence accession HQ14704 (Aiko and Mehta, 2013b). The fungal culture was maintained on potato dextrose agar slant at 4 °C and used for further experiments.

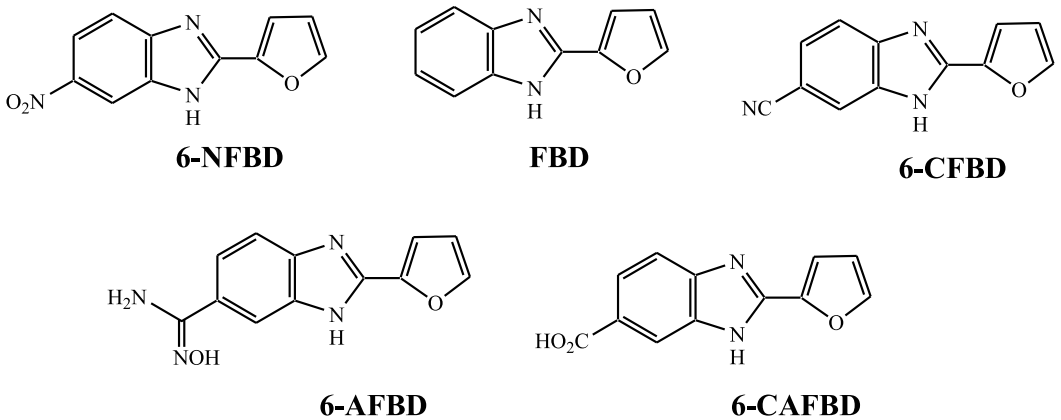

Fig. 1. Structures of Benzimidazoles. FBD: 2-(2-Furyl)benzimidazole; 6-NFBD: 2-(2-Furyl)-6-nitrobenzimidazole; 6-CFBD: 2-(2-Furyl)benzimidazole-6-carbonitrile; 6-AFBD: 2-(2-Furyl)benzimidazole-6-carboxamidoxime; 6-CAFBD: 2-(2-Furyl)benzimidazole-6-carboxylic acid.

2.2.1. Evaluation of fungal growth and aflatoxin B_1 production in presence of test compounds

The growth of *A. flavus* and AFB_1 production were studied in the presence of test compounds in the culture medium. Stock solutions of each test compounds (50 mg/mL) were prepared in Dimethyl sulfoxide (DMSO) and then diluted to 50 μg/mL of concentration in the yeast extract sucrose (YES) medium. The spores of *A. flavus* were harvested from the 9-day old culture on potato dextrose agar and counted using a hemocytometer. Fifty μL of spore suspension (10^5 spores/mL) was then inoculated into each flask containing 10 mL of YES medium. The flasks were incubated at 27 ± 2 °C for a period of 7 days. At the end of the incubation period, the fungal mass in terms of Dry Mycelia Weight (DMW) and AFB_1 production in spent media was determined. The mycelium was harvested by filtration using a Whatman filter paper and the broth obtained was used for the extraction of AFB_1. The wet weight of the mycelium was recorded and then dried at 60 °C in a hot air oven until it reached a constant weight and it was considered as DMW. The spent media was extracted with an equal volume of chloroform. The extraction was done thrice by wrist action using a separating funnel and kept for phase separation. The lower chloroform layers were then pooled, concentrated and analyzed by thin-layer chromatography, high-performance liquid chromatography (HPLC), and fluorescence spectroscopy for AFB_1.

2.2.2. Thin-layer chromatography (TLC)

Qualitative analysis of AFB_1 using thin-layer chromatography (TLC) was performed along with a standard AFB_1 (Supelco, Bellefonte, PA, USA). Ten microliters of the chloroform extract from the spent YES medium was applied on activated TLC plate (silica gel on aluminum foil Fluka, Munich, Germany) and chloroform-acetone mixture (85:15) was used as a mobile phase. After developing the chromatogram, the plates were viewed under UV light (365 nm) to detect the characteristic fluorescence and R_F value of AFB_1. The R_F value of spots was then compared with standard AFB_1.

2.2.3. Fluorescence spectroscopic analysis

The fluorometric analysis was carried to qualitatively analyze the emission peak for AFB_1. The instrument used was HITACHI F-7000 fluorescence spectrophotometer equipped with a xenon lamp of both the excitation at 365 nm and emission at 430 nm, and the slit width was set at 5.0 nm. Instrumental parameters were controlled with FL Winlan software. All samples were dissolved in chloroform.

2.2.4. High-performance liquid chromatography (HPLC) analysis

AFB_1 was quantified using reversed-phase high-performance liquid chromatography (YOUNGLIN INSTRUMENT Acme 9000) using C-18 column as per the standard supelco protocol. The chloroform extract was evaporated to dryness and the residue was re-dissolved in benzene. 20 μL of the sample was injected into the HPLC sampler and acetonitrile: water: methanol (30:50:20 v/v/v) was used as mobile phase at a flow rate of 1 mL/min. AFB_1 was detected with a UV detector at 365 nm wavelength. The amount of AFB_1 was determined from the standard and calculated as given (Aiko and Mehta, 2013b).

AFB_1 = (Peak area of sample ÷ Peak area of AFB_1 standard) × Concentration of standard

2.2.5. Determination of IC_{50} concentration of 6-NFBD and FBD

To determine the IC_{50} values for inhibiting the synthesis of AFB_1 in YES medium, different concentrations of test compounds (6-NFBD and FBD), ranging from 2 to 50 μg/mL were tested by the following procedure mentioned in section 2.2.1. AFB_1 level was quantified using HPLC analysis and inhibition percentage of AFB_1 synthesis was calculated following formula (Aiko and Mehta, 2013b).

Inhibition % = (1- Mean concentration of AFB_1 in treatment ÷ Mean concentration of AFB_1 in control) × 100.

2.2.6. Determination of anti-aflatoxigenic activity of test compounds in rice

White polished rice that had no fungal or AFB_1 contamination was used for the experiment. Ten grams of rice was taken in a 100 mL Erlenmeyer flask, and washed with distilled water and drained completely. Fifty μg/g of each compound was added separately to the washed rice and autoclaved at 121 °C for 20 min. The moisture content of the rice at the time of inoculation was maintained at 20–25%. An aliquot 100 μL of *A. flavus* spore suspension (10^5 spores/mL) was inoculated into the flask and incubated at 27 °C ± 2 °C for 7 days (Aiko and Mehta, 2013b). After incubation, AFB_1 was extracted from the rice culture directly with 30 mL chloroform by vigorous shaking and then filtered using Whatman paper. Qualitative and quantitative analysis of AFB_1 were carried out using TLC, Fluorescence spectrophotometry and HPLC.

2.2.7. Determination of effective concentration of 6-NFBD and FBD in rice

To find the effective concentration for inhibition of AFB_1 production in rice, different concentration of test compounds (6-NFBD and FBD) were taken, ranging from 10 to 50 μg/g and then tested according to the procedure mentioned in above section 2.2.6. AFB_1 level was quantified by HPLC analysis and percent inhibition was calculated following the formula mentioned in section 2.2.5.

2.2.8. Study of specific inhibition of aflatoxin B_1 synthesis by test compounds

In order to confirm test compound's effect on specific alteration of the secondary metabolism, each test compound at 50 μg/mL concentration was added to the culture after three days of incubation (before sporulation). Initially, the experiment was set up as described in above Section 2.2.1 without the addition of the test compounds. On the 4th day of incubation and when white mycelium covered the entire medium, the test compounds were added to the flask except for control flask in a sterile condition and the incubation was continued up to 7 days under the same conditions as mentioned above. The antifungal activity was determined for test compounds after 7 days incubation in terms of Dry Mycelium Weight (DMW) and AFB_1 production.

2.3. Gene expression studies

Spores of *A. flavus* were harvested from the 9-day old culture on potato dextrose agar and counted using a hemocytometer. Fifty μL of spore suspension (10^5 spores/mL) was inoculated in 10 mL of YES medium for control as well as test flask containing 50 μg/mL NFBD. The flasks were incubated at 27 ± 2 °C. After 7 days of incubation, the mycelium was harvested by filtration using a Whatman filter paper for gene expression studies. The mycelium was then washed with PBS buffer and stored at −80 °C for further use.

2.3.1. Total RNA extraction

Disruption of fungal mycelia (50 mg) was done using mortar and pestle method, and the total RNA extraction was carried by RNeasy Plant Mini Kit (Qiagen) according to the instructions given by the manufacturers. Total RNA obtained was diluted with 50 μL of RNase free water and stored at −80 °C for reverse transcription. Isolated RNA was quantified by absorbance at 260 nm and the purity was assessed using absorbance ratio A260/A280 measured using Nanodrop spectrophotometer (Eppendorf BioSpectrometer®, USA), and a final RNA concentration was adjusted to 300 ng/μL.

2.3.2. RT-PCR assay

RT-PCR was used to amplify the structural gene *aflB* and the regulatory gene *aflR* of AFB_1 biosynthetic pathway as target genes, and kept the *gpdA* gene as a housekeeping gene.

Table 1
Sequences of the nucleotide primers used in this study.

Primer code	Gene	Primer Sequence (5′→3′)	PCR Product Size (bp)
AflR-1F	***aflR***	ATGGTCGTCCTTATCGTTCT	**627**
AflR-2R		CCATGACAAAGACGGATCC	
AflB-1F	***aflB***	ACAATCGAATGACAACACTGC	**580**
AflB-2R		CCACCGAATCCACTACCTACA	
GPD-1F	***gpdA***	GACATCAACGTCCTCTCTAA	**356**
GPD-2R		GTCTTCTTGATGTCCTCGTA	

2.3.3. PCR primers

The primer sequence of *aflR* and *aflB* were shown to be involved in the AFB_1 biosynthetic pathway (Chang et al., 2005). The primer pair of the *gpdA* housekeeping gene was designed based on the genomic data of the *Aspergillus flavus* strain NRRL3357 (GenBank accession number XM_002373980) (Table 1). All primers were synthesized by GeNoRime (a division of Shrimpex Biotech Services Pvt Ltd, Chennai, India).

2.3.4. cDNA synthesis

Total RNA (2 µL) was used as a template to synthesize the first-strand cDNA according to the Omniscript Reverse Transcription Kit (Qiagen) instructions given by the manufacturer and finally used for qPCR.

2.3.5. Analysis of the genes expression linked to aflatoxin B_1 biosynthesis

Gene expression analysis was performed using the Bio-Rad CFX96 RT PCR detection system (Bio-RAD, USA). Optimization was done using *gpdA* gene. The reaction mixture was set up in triplicate each 10 µL volume contains 5 µL of SYBR® Premix Ex Taq™ (Takara Bio Inc., Japan) used as a fluorescent dye, 800 nM of each primer, and 1 µL of cDNA template were taken in MicroAmp optical 96-well reaction plates and sealed with adhesive covers (Bio-Rad). Three-step optimal thermal cycling conditions were performed as follows: Initial denaturation at 95 °C for 5 min, and then 40 cycles of amplification at 95 °C for 10s, 50 °C for 10s, and 72 °C for 30s. A final extension at 95 °C for 10s, 60 °C for 10s, and 95 °C for 15s to obtain melt curve analysis. Data were analyzed with the software CFX Maestro ™ Software (Bio-Rad). The quantification of the expression of *aflB* and *aflR* genes was performed using the housekeeping gene *gpdA*. The gene expression ratio was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). This method permits calculation of the expression ratio of the target gene between a tested sample and its control sample.

2.4. Cytotoxicity assay for the test compounds

The HeLa cell line was obtained from NCCS Pune, India. MTT colorimetric method given by Mosmann (1983) and modified by Ben Trivedi et al. (1990) was used for the assay. In brief, the compounds were dissolved in DMSO and further diluted with Dulbecco's modified Eagle's medium (DMEM, HiMedia, India). A hundred microliters of this sample were then added to the 96 well microplate. HeLa cell suspension of 1×10^5 cells/mL (100 µL) was added to each well. The plate was incubated at 37 °C under 5% carbon dioxide and 95% atmospheric air. The DMSO concentration was kept below 0.1% in the samples. Observations were taken after 48 h of incubation. The cell growth was measured in terms of color development due to the formation of formazan from a reduction of MTT by live cells. The absorbance was measured with a microplate reader (Bio-Rad 680, USA) at 540 nm with a reference wavelength of 600 nm. The percentage cytotoxicity was calculated as given by Panda and Mehta (2013):

Cytotoxicity % = (1- absorbance of treated wells ÷ absorbance of untreated wells) × 100

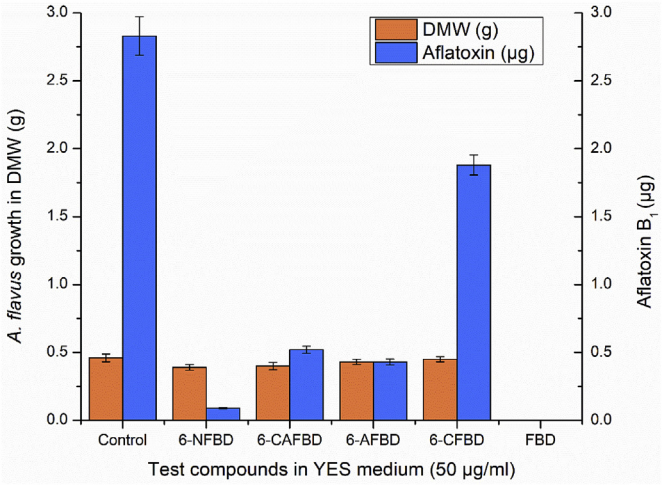

Fig. 2. Influence of test compounds on growth of *A. flavus* and aflatoxin B_1 production in 10 mL of YES medium.

2.5. Statistical analysis

In this study, the experiments were carried out in triplicate and repeated thrice to confirm the results. The data were reported as mean ± standard deviation and significant difference between mean values was determined by one-way analysis of variance (ANOVA) at $P < 0.05$.

3. Results

3.1. Growth and aflatoxin B_1 production by A. flavus

A luxuriant growth of *A. flavus* and AFB_1 production was noted in the control flask. In the presence of FBD, a known antifungal compound (Alasmary et al., 2015), complete inhibition of fungal growth at 50 µg/mL concentration was observed. In contrast to FBD, other benzimidazoles of 6-NFBD, 6-AFBD, 6-CAFBD, and 6-CFBD did not show much inhibition to fungal growth (Fig. 2). However, AFB_1 synthesis was significantly inhibited. The qualitative analysis with thin layer chromatogram (Fig. 3) showed a fluorescent spot corresponding to AFB_1 only in lane C (control) and 4 (6-CFBD). The fluorescence spectroscopic analysis (Fig. 4) of these samples showed typical fluorescence peak of AFB_1 at 430 nm in the control sample and 6-CFBD. Other samples did

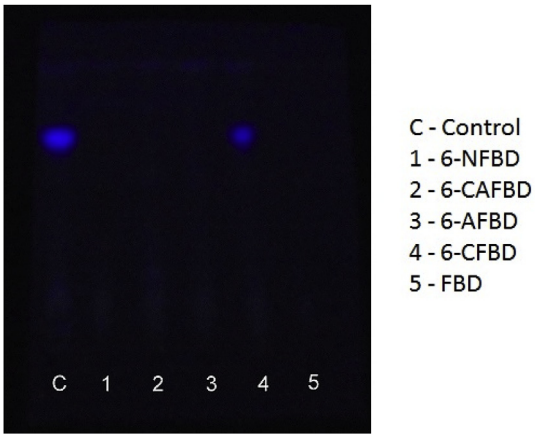

Fig. 3. Thin layer chromatogram of chloroform extracts of spent medium after *A. flavus* growth in presence of test compounds showing blue fluorescent aflatoxin B_1 spots from control and treated samples (C and 1–5). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

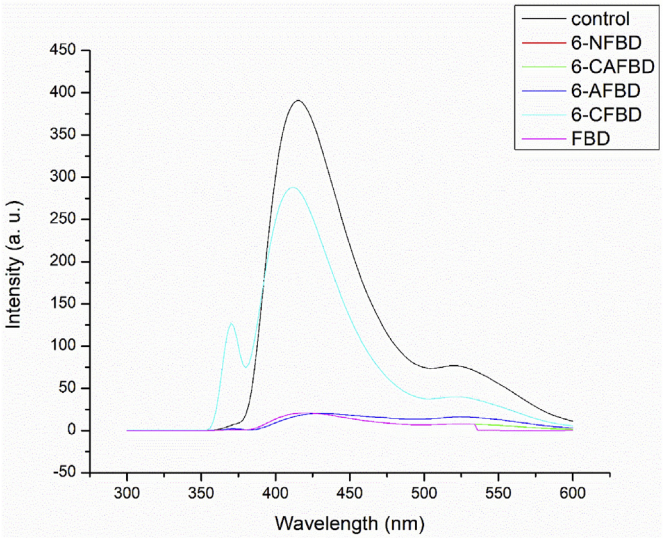

Fig. 4. Fluorescence spectrum of chloroform extracts of spent medium after *A. flavus* growth in presence of test compounds showing aflatoxin B_1 peak at 430 nm.

not show AFB_1 peak, confirming the observations of TLC analysis. The quantitative analysis from HPLC showed that 6-CAFBD inhibited less than 13% fungal growth but AFB_1 production was reduced by 81%. Similarly, 6-AFBD and 6-NFBD inhibited 84 and > 95% AFB_1 synthesis, respectively (Fig. 2). These data show that 6-NFBD, 6-AFBD, and 6-CAFBD specifically inhibiting the AFB_1 synthesis by hindering secondary metabolism of polyketide biosynthesis pathway. However, 6-CFBD found to be less effective compared to other compounds used, as it inhibited fungal growth only by 2% and AFB_1 synthesis by 33%.

3.2. Minimum inhibitory and IC_{50} concentration of 6-NFBD and FBD in culture medium

As the highest inhibition of AFB_1 was noted in the presence of test compounds FBD and 6-NFBD, further studies were carried out to determine the IC_{50} value and minimum inhibitory concentration. Fig. 5 shows the correlation between the concentration of the compound and the inhibition of AFB_1. The IC_{50} value obtained from the graph is 9.49 and 9.67 µg/mL for FBD and 6-NFBD respectively. The concentration of

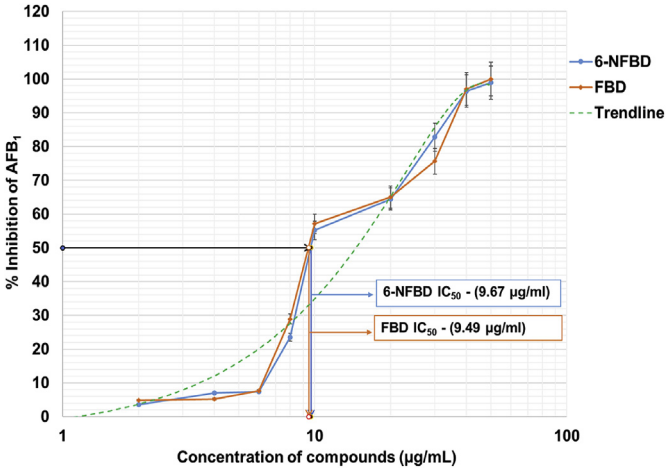

Fig. 5. Correlation between concentration of test compounds (2-(2-Furyl) benzimidazole (FBD), 2-(2-Furyl)-6-nitrobenzimidazole (6-NFBD) and inhibition of aflatoxin B_1 synthesis in YES medium. Graphical representation of IC_{50} values of 6-NFBD and FBD.

FBD or 6-NFBD at 40 µg/mL and above gives more than 95% inhibition, whereas 50 µg/mL exhibits 99% inhibition of AFB_1 synthesis.

3.3. Anti-aflatoxigenic activity of test compounds in rice

Experiments were performed on rice to find the practical application of test compounds to control fungal growth and AFB_1 synthesis. In the experiment, it was found that a dose of 50 µg of test compound per gram of rice delayed the growth of *A. flavus* by 2 days. However, the fungus started to grow from the 4th day. At the end of incubation period i.e. the 7th day, white fungal growth was noted in all the test flasks (Fig. 6). In contrast to the test flasks, the rice in the control flasks had mycelium growth and turned green due to spore formation. The amount of AFB_1 produced in the presence of test compounds and control is shown in Fig. 7. Similar to the observation in YES medium the test compounds did not inhibit the fungal growth but the AFB_1 synthesis was inhibited very effectively as in the case of 6-NFBD, 98% inhibition was noted. Inhibition by other compounds ranged between 50 and 95%. In contrast to study in YES medium, FBD did not show complete inhibition of fungal growth in rice, however, AFB_1 synthesis was inhibited by 98%.

3.4. The effective concentration of 6-NFBD and FBD in rice

Since the test compounds, 6-NFBD and FBD showed more than 95% inhibition in the synthesis of AFB_1 from *A. flavus* at 50 µg/g in rice, further studies were carried out with lower concentrations to determine the IC_{50} values (Table 2). A gradual inhibition in AFB_1 synthesis was observed with increasing concentration of test compounds. The IC_{50} values of 6-NFBD (23 µg/g) and FBD (22 µg/g) were noted. These are higher than the IC_{50} found in YES (9.49 and 9.67 µg/mL, respectively).

3.5. Specific inhibition of aflatoxin B_1 production

The addition of test compounds to the culture after three days of vegetative growth showed that DMW from test flask is almost similar to that in control (Table 3), but AFB_1 production was inhibited in comparison to the control. Addition of FBD and 6-NFBD at the concentration of 50 µg/mL of medium showed about 98 and 96% decrease in AFB_1 content. 6-CAFBD showed a 79% reduction in AFB_1. In the presence of 6-AFBD and 6-CFBD, only 47 and 34% reductions in AFB_1 production were noted when compared to the control. The chromatographic analysis of the culture extracts showed diminishing fluorescence of the AFB_1 spot on TLC. These results were also confirmed by the fluorescence spectroscopic analysis.

3.6. Analysis of the genes expression linked to aflatoxin B_1 biosynthesis

To confirm the observed changes in AFB_1 biosynthesis in the presence of benzimidazole, the change in expression of genes involved in AFB_1 biosynthesis Fluorescence. Expression of a regulatory gene *aflR* and a structural gene *aflB* was compared with respect to housekeeping gene *gpdA* using real-time PCR. *gpdA* expression in the presence and absence of NFBD was the same; however, expression of *aflR* and *aflB* gene was strongly downregulated in the presence of 6-NFBD (Fig. 8).

3.7. Effect of test compounds on HeLa cell lines

Preliminary safety studies of the test compounds were carried out using *in-vitro* tests on the animal cell line. For this purpose, HeLa cells were incubated for 48 h with a test compound and the cell proliferation was checked in terms of blue formazan formation from the reduction of MTT by live cells. The color intensity measured by colorimeter is proportionate to the number of live cells in the sample. Control well showed confluent HeLa cells after 48 h incubation. However, all cells were found dead in the presence of 8 µg/mL AFB_1 indicating high

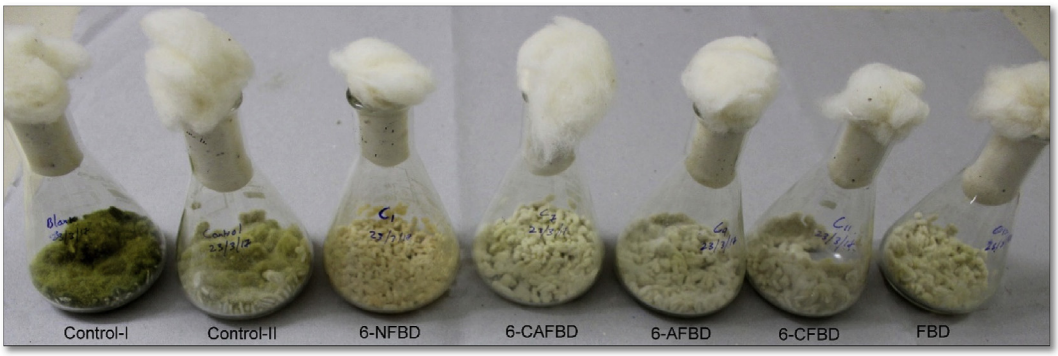

Fig. 6. Growth of *A. flavus* in presence of test compounds in rice on 7th day of incubation.

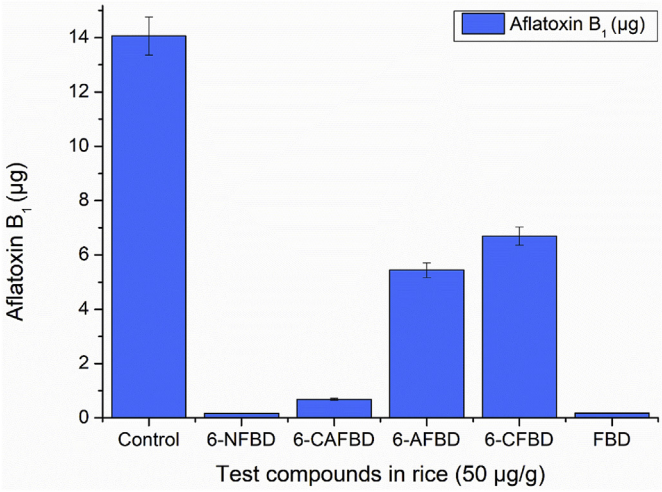

Fig. 7. Influence of test compounds on production of aflatoxin B_1 in rice. *A flavus* cultured in rice in presence of test compounds (50 µg/mL) for 7 days.

Table 2
Effective concentration of 6-NFBD and FBD for inhibition of aflatoxin B_1 in rice.

Compound	Concentration (µg/g)	Total AFB_1 produced in rice[b] (µg)	% Inhibition
Control	–	15.59 ± 0.15[a]	0
6-NFBD	10	15.23 ± 1.02[a]	2.30
	30	3.88 ± 0.33[a]	75.11
	50	0.19 ± 0.11[a]	98.78
FBD	10	15.02 ± 1.16[a]	3.65
	30	3.78 ± 0.13[a]	75.75
	50	0.18 ± 0.09[a]	98.84

Values are mean (n = 3) ± SD.
SD, standard deviation.
[a] Means with the same superscript in the same column for each variable showing no significant difference at ($P \leq 0.05$).
[b] Rice −10 g.

cytotoxicity of AFB_1 which is used as a positive control. The test wells of FBD, 6-CFBD, and 6-NFBD showed fully confluent healthy cells indicating no adverse effects on HeLa cells at the concentration of 100 µg/mL 6-AFBD well showed less growth when compared to the control but the cells were growing and appeared healthy (Fig. S1, Table 4). Therefore, these preliminary experiments showed that the test compounds are not toxic to HeLa cells at 100 µg/mL concentration, and to ensure the safe usage of test compounds, further experiments on animals are required.

Table 3
Aflatoxin B_1 Synthesis from *A. flavus* in YES medium when Test Compounds are added after 3 days of culture.

Compound (50 µg/mL)	DMW (g)	% Inhibition	Total AFB_1 produced in YES medium[b] (µg)	% Inhibition
Control	0.37 ± 0.08	0	2.85 ± 0.12[a]	0
6-NFBD	0.32 ± 0.14	11.55	0.11 ± 0.09[a]	96.11
6-CAFBD	0.34 ± 0.16	6.98	0.58 ± 0.15[a]	79.50
6-AFBD	0.37 ± 0.15	−1.34	1.49 ± 0.21[a]	47.63
6-CFBD	0.39 ± 0.13	−6.18	1.86 ± 0.12[a]	34.62
FBD	0.31 ± 0.11	16.66	0.03 ± 0.02[a]	98.84

Values are mean (n = 3) ± SD.
SD, standard deviation.
[a] Means with the same superscript in the same column for each variable showing no significant difference at ($P \leq 0.05$).
[b] YES medium-10mL.

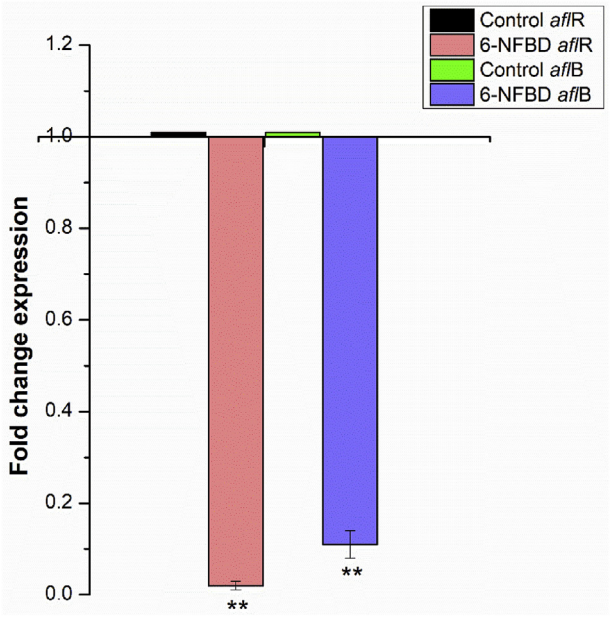

Fig. 8. Effect of NFBD on the expression of aflatoxin B_1 synthesis regulatory gene *afl*R and structural gene *afl*B. **p-value < 0.01.

4. Discussion

The anti-aflatoxigenic activity is generally thought to be the outcome of the fungicidal action, and it was clearly demonstrated by the direct correlation between the degree of fungal growth and aflatoxin production (Ni et al., 2010). Benzimidazoles are known to exert

Table 4
Effect of test compounds on HeLa cells after 48 h of culture in DMEM medium and 10% FBS.

Compound	μg/well	% Cytotoxicity
Control	–	0
AFB_1[b]	0.8	94.24 ± 1.00[a]
6-NFBD	1	−8.17 ± 0.78
	10	−4.29 ± 0.80
6-CAFBD	1	1.83 ± 0.58[a]
	10	3.28 ± 0.34[a]
6-AFBD	1	7.46 ± 0.46[a]
	10	9.38 ± 0.72[a]
FBD	1	−32.61 ± 1.07
	10	0.57 ± 0.63[a]

Values are mean (n = 3) ± SD.
SD, standard deviation.
[a] Means with the same superscript in the same column for each variable showing no significant difference at ($P \leq 0.05$).
[b] Standard AFB_1Used as a positive control.

fungicidal effect and more commonly studied against pathogenic strains like *Candida albicans,* and *Aspergillus fumigatus* (Del Poeta et al., 1998). Generally antifungal agents work by impairing or disrupting the ergosterol, chitin synthesis, targeting nucleic acids, protein synthesis and microtubule synthesis (Kathiravan et al., 2012), and however the mechanism of fungicidal activity of benzimidazoles is not yet clear (Can et al., 2017; Thamban Chandrika et al., 2018). Apart from pathogenic fungi, these compounds are also found to be effective against saprophytic strains like *Aspergillus flavus* (Chandrika et al., 2016). Moon et al. (2016) have reported that 1,3-benzodioxole and compounds containing methylenedioxy are fungicidal at 1000 μg/mL concentration. However, the activity decreased drastically at lower concentrations. In the present study, we found complete inhibition of fungal growth by FBD at 50 μg/mL and at 9 μg/mL 50% inhibition was noted. More polar derivatives of FBD (6-NFBD, 6-AFBD, 6-CAFBD, and 6-CFBD) did not impair the fungal growth but effectively inhibited the AFB_1 biosynthesis.

While studying the gene expression of *A. flavus* in control and in the presence of 6-NFBD, a significant down-regulation of regulatory *aflR* and structural *aflB* gene was observed. 6-NFBD, a strong AFB_1 inhibitor did not show any effect on the expression of the housekeeping gene *gpdA*. The same level of *gpdA* expression was noted in the presence or absence of 6-NFBD. This shows that 6-NFBD does not alter the primary metabolism of *A. flavus*. *aflR* gene is a positive regulator for many genes involved in the secondary metabolism, especially genes coding for the enzymes involved in AFB_1 synthesis cascade. Absence of *aflR* gene product resulted in the inhibition of aflatoxins (Al-Saad et al., 2016; Liu and Chu, 1998; Yu et al., 2004), and we observed the down-regulation of *aflR* in presence of 6-NFBD that can be correlated with the down-regulation of *aflB*. The structural gene for the beta subunit of fatty acid synthase enzyme part of the gene cluster that mediates AFB_1 biosynthesis. These observations can explain the inhibition of AFB_1 in presence of 6-NFBD without having any adverse effect on the fungal growth.

It was reported that the membrane permeability pattern of a different set of benzimidazoles correlates with the polarity and hydrophilicity factors (Alvarez-Figueroa et al., 2011). Therefore, our results predict that such polar derivatives of FBD are controlling the AFB_1 synthesis.

It is noted from many studies that compounds found to be effective in laboratory conditions, like culture medium, have failed in the field when tested with food grains. However, these benzimidazole derivatives used in the study have also shown good anti-aflatoxigenic activity in rice. Though the IC_{50} concentrations were comparatively higher than that in YES medium (Fig. 5). The test compounds utilized in this study were found to be quite stable in nature even during autoclaving along with culture medium (YES) or rice that is done to ensure sterility. Hence these compounds can be added prior to processing or during storage as antifungal or anti-aflatoxigenic agents.

Many fermentation processes are carried out by species of *Aspergillus* and *Penicillium* having the potential to synthesize mycotoxins. Not only the organisms involved but contamination carried with raw material also has the chance to grow due to suitable physiological and nutritional conditions and produce mycotoxins including aflatoxins during the fermentation process. *Aspergillus oryzae,* the main organism for koji fermentation is an atoxigenic strain of *A. flavus* developed by domestication having 99.5% genomic similarity including the genes involved in AFB_1 biosynthesis (Gibbons et al., 2012; Yin et al., 2018). Though this strain does not produce AFB_1, longer incubation may result in mycotoxin synthesis (Ciegler and Vesonder, 1987). Long ago, Kinosita et al. (1968) reported the toxigenic strains of *A. flavus* and other *Aspergillus* genus from koji.

Cheese is another common fermented product, which has the risk of mycotoxin contamination. Hymery et al. (2014) in their comprehensive review on mycotoxins in cheese stated that even though the risk of AFB_1 contamination is low in cheese, it should be controlled. Cheese is contaminated with various toxigenic fungi including AFB_1-producing strains of *Aspergillus* but it is a poor substrate for AFB_1 production. Apart from AFM_1, other aflatoxins had not been reported from cheese, but there were other reports of sterigmatocystin contamination (Scott, 1989; Nasser, 2001), which is a precursor and structurally similar to aflatoxins. However, it is less toxic than aflatoxins (Sweeney and Dobson, 1998).

The mycotoxin risks in the fermented food can be prevented with the use of benzimidazole derivatives 6-NFBD, 6-AFBD and 6-CAFBD as established through this study. These compounds impaired the AFB_1 synthesis without affecting the mold growth. Since mold growth is necessary for the fermentation process, these compounds may be better candidates as AFB_1 inhibitors when compared to other known fungicides. Safety is an important aspect for the compounds to be used in food processing. Many benzimidazole derivatives are already in use as therapeutics, and our preliminary assay with animal cell line also showed of their non-toxic nature, at the concentrations used, making them promising candidates to be used for the control of AFB_1 during food processing. However, more animal experiments are necessary to confirm the safety of these compounds.

In conclusion, present study reports that test compounds; 6-NFBD, 6-AFBD and 6-CAFBD derivatives of a known fungicide, FBD, do not impair the growth of *A. flavus*, but specifically inhibit the biosynthesis of AFB_1. Test compounds are active even after autoclaving with YES medium or rice showing their thermal stability. *In-vitro* testing on HeLa cell line showed no toxic effect of the compounds at the concentrations 10 times higher than used in present study. Due to their non-toxic nature, thermal stability and specific inhibition of AFB_1 synthesis, these compounds can be added during fermentation, food processing or storage as anti-aflatoxigenic agents.

Conflicts of interest

The authors declare that they have no conflict of interest in the publication.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.toxicon.2019.09.018.

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