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Is Aflatoxin B1 A Biomarker for Pathogenic Potential of *Aspergillus flavus*?

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Abstract

Given the opportunity and the host immune status, *Aspergillus flavus* can produce aspergillosis affecting various body organs, while its toxin, Aflatoxin B1 (AFB1) has been implicated as a carcinogen in hepatocellular carcinoma. Based on previous findings, *A. flavus* can be divided into two groups, (i) isolates that can synthesize AFB1, and (ii) isolates unable to produce AFB1. The aim of this study was to assess whether AFB1 can be used as a marker to differentiate clinical and non-clinical isolates of *A. flavus*. Representative clinical isolates were obtained from patients while non-clinical isolates were obtained from the environment. Isolates were identified as *A. flavus* using selective media. AFB1 production was assessed through cultural assays and genes for aflatoxin production, aflR and aflS, were amplified using PCR. Conditioned media and methanol extract of *A. flavus* isolates were prepared and tested for AFB1 presence using Liquid Chromatography-Mass Spectrometry (LC-MS). Additionally, conditioned media and extract were tested for their cytotoxic effects on primary human brain microvascular endothelial cells (HBMEC) and immortalized human heptaoma cells (Huh7). Both clinical and non-clinical isolates of *A.flavus* exhibited aflatoxin production, albeit some clinical isolates produced excessive AFB1 (up to 15785 ng/mL). Importantly, *A. flavus* isolates produced higher levels of AFB1, exhibited increased host cell cytotoxicity, whereas strains exhibiting negligible amount of aflatoxin exhibited minimal cytotoxic effects suggesting AFB1 as a marker for pathogenic potential of *A. flavus*. The ability of aflatoxigenic *A. flavus* to produce host cell death in primary cells raises additional concern for patients suffering from *A. flavus* infection.

Keywords: Aflatoxin B1 (AFB1); *Aspergillus flavus;* Clinical and non-clinical isolates; HBMEC; Huh7

Introduction

Aspergillus flavus is a saprotrophic fungus that is widely distributed in the environment. Given the access and the host immune status, *A. flavus* can be pathogenic for humans and may result in different types of aspergillosis such as allergic bronchopulmonary aspergillosis, invasive aspergillosis or aspergilloma, each affecting the body in different ways [1,2]. Additionally, *A. flavus* is the major species responsible for the production of aflatoxins. Among different types of aflatoxins, aflatoxin B1 (AFB1) has been regarded as one of the most potent carcinogen in hepatocellular carcinoma [3]. Contamination of commonly consumed food items such as maize, nuts, rice, wheat, and spices like chillies, black pepper, turmeric result in chronic exposure to the toxin which may lead to liver cancer [1,2]. An indirect source of exposure may also be through animals which have ingested contaminated feed. As aflatoxins are highly liposoluble, they are readily absorbed through the gastrointestinal tract [4]. Globally, approximately 4.5 billion persons living in developing countries are frequently exposed to unchecked amounts of the mycotoxin [5]. Regions such as the sub-Saharan Africa, China, and Southeast Asia are more susceptible to aflatoxin-related hepatocellular carcinoma because of favorable temperatures for fungal growth and poor processing of aflatoxin-contaminated foods. A gene cluster of 30 genes encodes for aflatoxin biosynthesis. These genes are clustered within a 75kbp region on chromosome 3 of the fungal genome [6]. The major regulatory gene is *aflR*, and is located in the center of the cluster and is needed for the transcriptional activation of structural genes thereby acting as a positive regulator of expression of AFB1. Adjacent to *aflR*, is a divergently transcribed gene called *aflS* which is also involved in the regulation of transcription [7-9]. Based on previous findings, *A. flavus* can be divided into two groups, (i) isolates that can synthesize AFB1, and (ii) isolates unable to produce AFB1 [10,11]. The aim of this study was to assess whether AFB1 can be used as a marker to differentiate clinical and non-clinical isolates of *A. flavus* as well as to determine the usefulness of the assays to determine aflatoxin production.

Materials and Methods

Fungal cultures

Nine fungal cultures were obtained for the present study (Table 1). Non-clinical isolates (S1, S2, S3 and S4), sourced from the environment, were provided by M. Sohail and N. Ikram (University of Karachi). Clinical isolates (C1, C2, C3, C4 and C5) were provided by E. Khan from clinical microbiology laboratory at the Aga Khan University. All cultures were grown on Potato Dextrose Agar (PDA) (Oxoid) at 29°C for 5 days. For toxin extraction, cultures were grown in Sabouraud's broth (Glucose 8 g, Peptone 2 g, water 100 ml) at 29°C for 5 days in a shaking incubator [12]. Additionally, *Fusarium solani* was used as a control in PCR and was purchased from First Fungal Culture Bank of Pakistan (FCBP0055). It was grown in Sabouraud's broth at 29°C for 5 days in a shaking incubator.

Human brain microvascular endothelial cells (HBMEC)

Primary HBMEC were grown in tissue culture flasks in Roswell Park Memorial Institute Medium (RPMI-1640) containing 10% heat-

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inactivated foetal bovine serum, 2 mM glutamine, 1 mM Na-pyruvate, 100U penicillin per mL, 100 µg streptomycin per mL, non-essential amino acids and vitamins as previously described [13]. For assays, HBMECs were seeded in 24-well plates at a density of 2×10^5 per mL in each well and plates incubated at 37°C in a 5% CO_2 incubator, which resulted in the formation of complete monolayers within 48 h. These primary HBMEC originated from small fragments of cerebral cortex derived from individuals who had undergone surgical resections for seizure disorder at Johns Hopkins University School of Medicine (USA) were used for HBMEC isolation. The primary HBMEC were positive for endothelial markers such as expression of F-VIII, carbonic anhydrase IV and uptake of acetylated low density lipoprotein (DiI-AcLDL) as previously described [13,14] and resulted in >99% pure endothelial cultures [15].

Human hepatocellualar carcinoma cells (Huh7)

Cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% heat-inactivated foetal bovine serum, 100U penicillin per mL, 100 µg streptomycin per mL and 2 µg ciprofloxacin per mL. For assays, Huh7 cells were seeded in 24-well plates at a density of 0.05 $\times 10^6$ per mL in each well and plates incubated at 37°C in a 5% CO_2 incubator, which resulted in the formation of complete monolayers within 48 h [16].

Culture assays and morphological characteristics of *Aspergillus flavus*

Fungal cultures were grown on selective and differential AFPA base medium (Oxoid) following incubation at 29°C for 5 days. Plate were inverted and observed for colonies having an orange underside, i.e., a specific characteristic of *Aspergillus flavus*. Additionally, fungal cultures were grown on Potato Dextrose Agar (Oxoid) plates following incubation at 29°C for 5 days. The reverse side of colonies was observed to check for secretion of yellow pigment into the semi-transparent medium, i.e., characteristic of aflatoxigenic *A. flavus* [1].

Biochemical assays

Fungal cultures were grown on Potato Dextrose Agar plates by incubation at 29°C for 5 days. Plates were inverted and few drops of 100% ammonium were placed on the inside of the lids and the underside of colonies was observed for a colour change due to the ammonium hydroxide vapours, characteristic of aflatoxigenic *Aspergillus flavus* [1].

Aflatoxin extraction and Liquid Chromatography-Mass Spectrometry (LC-MS) quantification

For toxin extraction, fungal cultures were grown in Sabouraud's broth (Glucose 8 g, Peptone 2 g, water 200 mL) at 29°C for 5 days in a shaking incubator [12]. The supernatant (conditioned media) was collected by centrifugation at 4500 × g for 30 min and passed through filter with a pore size of 0.2 µm and used for subsequent assays.

For LC-MS, 100 mL of conditioned media was incubated with 50 mL of chloroform and kept in a shaker for 30 min. The total mixture was passed through a plastic separator where chloroform separated to the lower end and approximately 40 mL was collected. It was left at room was isolated through separating funnel and lower layer comprising around 40 mL of chloroform was collected. It was left at room temperature in a petri dish under the hood for 2 h and the remaining 1 mL was collected in a glass vial. Next, chloroform was evaporated under reduced pressure using rotatory evaporator. The residual was dissolved in 1mL of chloroform and evaporated under the stream of nitrogen. Finally, samples were re-dissolved in a mixture of acetonitrile, methanol and water (with a ratio of 22.5:22.5:55), before subjecting it to LC-MS analysis. Samples and standards of aflatoxins were loaded in the autosampler (Surveyor Autosampler Plus) and 5 µL of them were injected to LC-MS/MS system (Finnigan LTQXL a Linear Quadrupole Ion Trap MS/MS equipped Surveyor HPLC system). Four species of aflatoxins (B_1, B_2, G_1 and G_2) have been chromatographed using Reverse Phase chromatography (column C_{18}, 4.6 × 50 mm, 3 µm particle size, manufactured by Phenomenex), at flow rate of 0.4 mL per min at isocratic solvent system (acetonitrile, methanol and water, ratio of 22.5:22.5:55, respectively). Aflatoxins were ionized using Electro Spray Ionization (ESI) probe at positive ion mode. ESI source parameters (probe position, source voltage 4 kV, sheath gas flow 2L/min, Aux gas flow 0.4 L/min, capillary temperature 275°C, capillary voltage 45 V and tube lens voltage 110 V), voltages of ion optics and MS parameters were optimized manually as well as by automatic and semi-automatic tuning process to get maximum signal strength of all the analytes in MS an MS^n mass spectra. Selected reaction monitoring approach (Table 2) was used for confirmation and quantification of the aflatoxins. Quantification of these analytes were done from the standards curves generated by external standards peaks areas (Figure 1).

The pellet (fungal mycelia) was dried and resuspended in 100% methanol, sonicated, and left on shaker overnight. The supernatant was collected after centrifugation at 14000 x g for 15 min and passed through filter with a pore size of 0.2 µm. Finally, one ml of methanol extract was collected in glass vials and subjected to LC-MS analysis as stated above.

DNA Extraction

Fungal DNA was extracted as previously described [17]. Briefly, one gram of mycelia was ground with acid washed sand in mortar with 5 mL of lysis buffer (10 mM Tris-HCl pH7.5, 0.5 M NaCl and 1% SDS). Then 6.6 mL of phenol: chloroform: isoamylalcohol (PCI 25:24:1) was added, and mixed completely and incubated at 65°C for 20 min. The suspension was centrifuged at 4°C for 30 min at 4500 x g and the aqueous supernatant was collected. Re-extraction was done with PCI (4:5, v/v) and the upper aqueous phase containing DNA was collected in a fresh tube. NaAcetate (pH 5.2) was added to a final concentration of 300 mM, followed by 0.7 volume of isopropanol. The precipitated DNA was collected by centrifugation at 14000 × g and washed twice, first with 70% ethanol and then with absolute ethanol. Next, the dry DNA pellet was dissolved in 30 uL of TE buffer (10 mM Tris-HCl pH 8.0, 1.0 mM EDTA) and its concentration was quantitated with Nanodrop ND-1000 Spectrophotometer (Thermo Scientific).

PCR based assay

PCR for aflatoxin producing genes (*aflR* and *aflS*) was done as previously described [12]. The primers used are listed in Table 3. As an internal control, 18S primers were used. PCR cycles were as follows: Initial denaturation was done at 95°C for 5 min, followed by 30 cycles consisting of denaturation at 95°C for 30 sec, annealing at 57°C (aflR) / 46°C (aflS) / 55°C (18S) for 30 sec, and extension at 72 for 1 min. The final extension was done at 72 for 5 min. The PCR products were electrophoresed on a 2% agarose gel and the expected band sizes were 400bp for *aflR*, 490bp for *aflS* and 359bp for 18S. PCR product from representative isolates of *A. flavus* was also sent to Macrogen, Korea for sequencing.

Sample	Source of Samples
C1	Clinical - Bronchial lavage fluids (patient with chronic cough; Asthma/TB/COPD/Allergic broncho pulmonary aspergillosis)
C2	Clinical - Bronchial lavage fluids (patient with chronic cough; Asthma/TB/COPD/Allergic broncho pulmonary aspergillosis)
C3	Clinical – Invasive fungal sinusitis in leukemia
C4	Clinical - Brain abscess
C5	Clinical - Nasal polyp (non-invasive disease usually associated with allergic rhinitis)
S1	Environmental – Growing as a contaminant on media
S2	Environmental – Growing as a contaminant on media
S3	Environmental – Grown as a contaminant on media
S4	Environmental – Isolated from the soil

Table 1: *Aspergillus flavus* isolates used in the present study.

Mycotoxins	Retention Times [min]	Nominal mass g/mol	Precursor ions [M + H]+	Collision Energy [V]	Product Ions [m/z]
AFB_1	15.69	312	313	25	285
AFB_2	13.34	314	315	25	287
AFG_1	12.00	328	329	25	311
AFG_2	10.33	330	331	25	313

Table 2: Chromatogram and mass spectra of aflatoxins standards.

Gene	Primer Sequence 5'-3'	
	Forward	Reverse
aflR	GCACCCTGTCTTCCCTAACA	ACGACCATGCTCAGCAAGTA
aflS	GGAATATGGCTGTAGGAAG	GGAATGGGATGGAGATG
18S	ATGGCCGTTCTTAGTTGGTG	GTACAAAGGGCAGGGACGTA

Table 3: Primer used for PCR in the present study.

Cytotoxicity assays

Cytotoxicity assays were performed as previously described [13]. Briefly, HBMEC and Huh7 were grown in 96-well plates and cells were exposed to crude fungal extracts (conditioned media or methanol extract) at a ratio of 1:10/well (15 µL in 150 µL). The solvent alone was used as a control. Plates were incubated at 37°C in a 5% CO_2 incubator for 24 h. Next, the supernatants were collected by centrifugation at 3000 × g for 8 min to remove cellular debris. Cytotoxic effects were determined by estimating the amount of lactate dehydrogenase released from the cells using a Cytotoxicity Detection kit (Roche Applied Sciences, Mannheim, Germany). The percent cytotoxicity was calculated as follows: % cytotoxicity = (sample value – control value) / (total LDH release – control value) X 100. Control values were determined by incubating HBMEC or Huh7 monolayers with RPMI-1640 or DMEM, respectively. The total LDH release was obtained by completely lysing the HBMEC and Huh7 by treating them with 1% Triton X-100 for 30 min.

Results

Culture assays demonstrated the presence of aflatoxin B1 (AFB1) in both clinical and non-clinical isolates of *Aspergillus flavus*

Both clinical (C1-C5) and non-clinical (S1-S4) isolates of *A. flavus* were tested for their potential to produce AFB1. First, the identity of *A. flavus* using selective culture assays. When grown on PDA plates, the colonies appeared olive to lime green with wooly to cottony textures and smooth to finely rough conidia. When grown on AFPA base, which is selective and the identification media for *A. flavus,* the underside of colonies appeared orange, confirming the cultures to be *A. flavus*. The isolates were then tested through cultural methods for AFB1 detection. When grown on PDA plates, the underside of colonies of C1, C5, S1, S2 and S3 showed strong yellow colour indicating AFB1 production [1]. Underside of colonies of C2, C3, C4 and S4 appeared pale white, lacking a strong yellow pigment production (Figure 2A). When these colonies were exposed to ammonium hydroxide vapours, only the yellow colonies of C1, C5, S1, S2 and S3 turned strongly pink indicating AFB1 production, while the colonies of C2, C3, C4 and S4 isolates did not change (Figure 2B).

PCR-based assays detected AFB1-producing genes, *aflR* and *aflS* in clinical and non-clinical isolates of *Aspergillus flavus*

The presence of AFB1 production was further tested by PCR amplification of AFB1 producing major genes, *aflR* and *aflS*. The *aflR* was amplified from all isolates except C3 (Figure 3A). In the case of *aflS* amplification, *aflS* was present in C3 as well as in all other isolates tested (Figure 3B). The DNA content was present equally in all isolates as indicated by 18S ribosomal unit amplification (Figure 3C). DNA from *Fusarium solani* was used as a specificity control and did not show amplification from *aflR* and *aflS* primers indicating that primers used were specific for the target genes.

The gene products were sequenced and multiple sequence alignment of *aflR* and *aflS* was done from representative clinical and non-clinical strains, C5 and S4, respectively. Alignment was done with known sequences of *aflR* and *aflS* genes from NCBI database (accession number FN398160.1 and AY510455.1, respectively). The results revealed that there was >99% sequence similarity in both clinical and non-clinical isolates and with original sequence present in the database (data not shown).

LC-MS quantification of aflatoxins in clinical and non-clinical isolates of *Aspergillus flavus*

Crude extract were prepared from clinical and non-clinical isolates of *A. flavus*, by growing them in liquid media. The quantity of aflatoxin present, in conditioned media and methanol extract, was assessed by LC-MS. The results revealed high amount of aflatoxin in extracts from C5, followed by C4 and S1 (Table 4). It was observed that apart from AFB1, some isolates also expressed other types of aflatoxin, i.e. B_2, G_1 and G2. Again, highest amount was observed in C5 isolate. The extract from C3 showed minimal amount of aflatoxin. In fact, it was not detected in methanol extract at all (Table 4). Extract from S4 also showed minimal aflatoxin production and its methanol extract showed complete absence of aflatoxin.

Methanolic extract of *A. flavus* but not conditioned media produced host cell death

Cytotoxic effects of conditioned media and methanolic crude extract of *A. flavus* on human cells were determined using primary HBMEC and immortalized Huh7 cells. When cells were exposed to methanol extract, almost all isolates exhibited HBMEC cytotoxicity, with C4 isolate exhibiting cell death at 53.2% ± 5.3 and C5 isolate at 56.9% ± 3.1 ($P<0.01^{**}$ using Student's t-test). The minimum cytotoxicity was seen by C3 isolate at 10.92 ± 3.2 and S4 isolate at 18.7 ± 1.5 (Figure 4A). In case of Huh7 cells, C4 isolate showed maximum cytotoxicity at 27.4 ± 3.7, followed by C5 isolate at 25.4 ± 3.3. Methanol extract from C1, C2 and S2 isolates exhibited high cytotoxic effects ($P<0.05^{*}$ and $P<0.01^{**}$ using Student's t-test) (Figure 4B).

When cells were exposed to conditioned media, isolate C4 showed

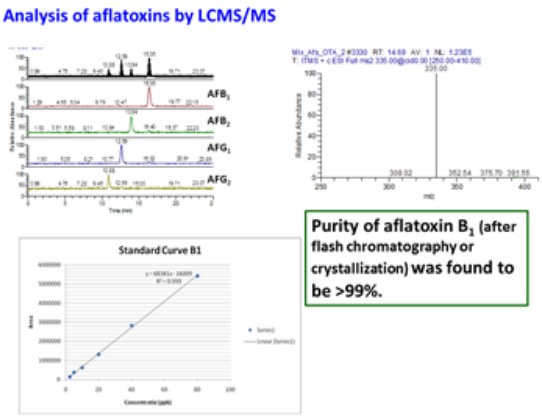

Figure 1: Aflatoxins were analyzed on LC-MS/MS (LTQXL, with Surveyor HPLC system) using ESI prove at positive ion mode employing selective reaction monitoring (SRM). Purity of aflatoxin B_1 (after flash chromatography or crystallization) was found to be >99%.

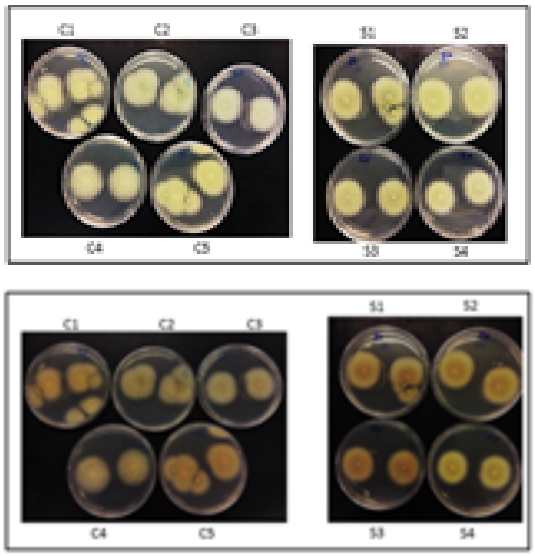

Figure 2: The presence of aflatoxin B1 (AFB1) in *Aspergillus flavus* isolates tested in the present study was determined using culture assays as described in Materials and Methods. Secretion of yellow pigment into the medium was observed on PDA plates by both clinical and non-clinical isolates **(A)**. Ammonium hydroxide vapour-induced colour change was observed in both clinical and non-clinical isolates of *A. flavus* **(B)**.

limited cytotoxicity i.e., 12.7% ± 4.3 (P>0.05 using Student's t-test) (Figure 5A). Same was the case with Huh7, where C4 isolate showed 9.2% ± 2 cytotoxicity (P>0.05 using Student's t-test) (Figure 5B).

Discussion

Economic losses due to aflatoxin contamination of major food crops such as corn, rice, wheat and nuts is a global problem [18]. Association of AFB1 with hepatocellular carcinoma has also been well documented [19]. Production of aflatoxins by *A. flavus* may vary upon environmental as well as on genetic factors. Our study reports a comparison of strains from clinical and environment settings for their aflatoxigenic properties. We also report the direct cytotoxic effects of crude extract originated from these isolates on primary HBMEC and immortalized Huh7 cells. The results obtained from culture assays indicate that even though some isolates did not show the characteristic features of aflatoxin production i.e., yellow pigment production and pink coloration of colonies upon exposure to ammonium hydroxide vapours, they were still producing aflatoxins when measured through LC-MS, suggesting the reliability, accuracy and sensitivity of LC-MS while culture assays may provide false negatives [20]. Thus there is a need for a distinct method/medium to differentiate aflatoxigenic *Aspergillus flavus* isolates and those producing no and/or minimal AFB1 and this will be the subject of future studies.

At the genetic level, *aflR* and *aflS* are major regulators for structural genes in the aflatoxin gene cluster. One of our clinical isolates C3, was not PCR positive for *aflR* (Figure 2A). There is a possibility of gene deletion from within clusters [21]. The absence of *aflR*, and thus lack of aflatoxin, was further corroborated by negative results in culture assays (Figure 1A and 1B), and no detection of aflatoxin in LC-MS (Table 4), as well as minimal cytotoxicity in functional assays (Figure 4A). The results indicate a larger amount of aflatoxin production from clinical isolates than environmental ones. From LC-MS data (Table 4), it was observed that C5 and C4 isolates expressed 15785 ng/mL and 1951.1 ng/mL AFB1 respectively, compared to environmental isolates. Our findings corroborate the work of Leema [22] where AFB1 was detected in significantly higher percentage of clinical samples isolated from keratitis infection as compared to isolates from the environment. Thus it is tempting to speculate that the host environment induces *A. flavus* to produce aflatoxin and the production of aflatoxin acts as a pathogenic factor and possibly affect the immune system. To this end, several lines of evidence suggest that AFB1 exposure affect the immune system by decreasing secretory Immunoglobulin A in saliva [23], a significant decrease in T and B cells [24], increased host susceptibility to bacterial and parasitic infections and decreased secretion of interleukin-1, interleukin-6 and tumor necrosis factor-alpha [25], in patients who have had chronic exposure to the toxin. Therefore, it is quite possible for the fungus to increase its production of aflatoxin in order to facilitate infection.

Apart from its toxic effects, it remains unclear as to why *A. flavus* makes the toxin as it is not required for the fungus to survive. It is

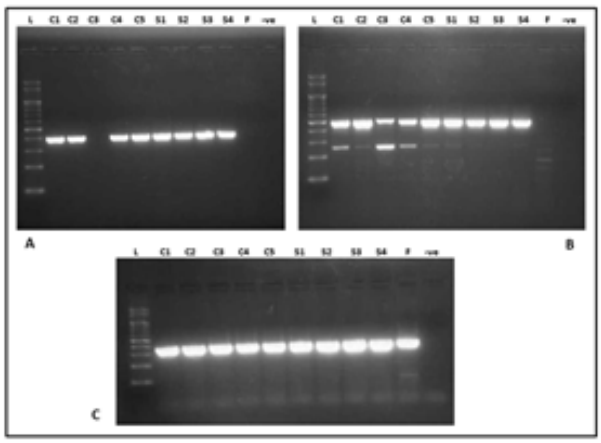

Figure 3: PCR-based assays to detect AFB1-producing genes in *Aspergillus flavus*. Two major genes (*aflR* and *aflS*) for AFB1 production were amplified by PCR. Both clinical and non-clinical isolates of *A. flavus* showed the presence of *aflR* (400bp) and *aflS* (489bp), except C3, where *aflR* was undetected (A and B). DNA from the fungus, *Fusarium solani* [F] was used as a primer-specificity control. Region of *18S* ribosomal DNA (359bp) was amplified as a positive control (C). Ladder used was of 100bp.

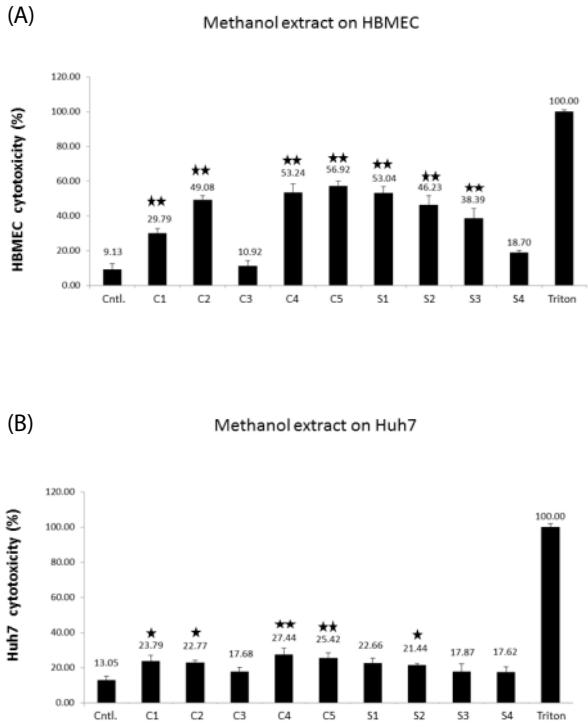

Figure 4: Human brain microvascular endothelial cells (HBMEC) and human hepatocellualar carcinoma cells (Huh7) were exposed to the methanolic extract of clinical and non-clinical isolates of *Aspergillus flavus* and LDH assays were performed to assess cytotoxicity. Note that all isolates tested showed host cell death as compared to control, except C3 and S4 which showed minimal host cell death (P<0.01** using Student's t-test) **(A)**. The isolates C1, C2, C4 C5 and S2 showed increased cell death as compared to control (P<0.05* and P<0.01** using Student's t-test) **(B)**. Equal volume of methanol was used as control. The results are presented as the mean ± standard error of three independent experiments performed in duplicate.

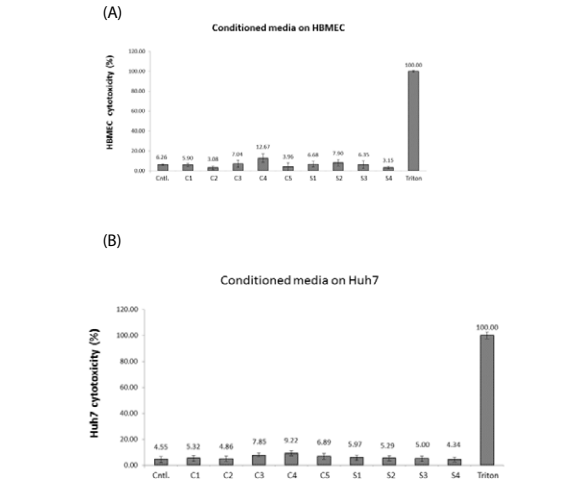

Figure 5: Human brain microvascular endothelial cells (HBMEC) and human hepatocellualar carcinoma cells (Huh7) were exposed to the conditioned media of clinical and non-clinical isolates of *Aspergillus flavus* and LDH assays were performed to assess cytotoxicity. Equal volume of Sabouraud's broth was used as a control. The results are presented as the mean ± standard error of three independent experiments performed in duplicate.

Extract	Samples Name				
		1	2	1	2
Chloroform extract from conditioned media of *A. flavus*	C1	269.8	10.81	7.379	0.215
	C2	805.3	39.9	26.04	0
	C3	17.53	0	0	0
	C4	1951.1	559	72.7	2.02
	C5	15785	1021.6	522	50.5
	S1	7002	275.5	274	12.5
	S2	710	30.1	33.5	0
	S3	31.5	0	0	0
	S4	12.9	0	0	0
Methanolic extract of *A. flavus* mycelia	MC1	4.30	0	0	0
	MC2	17.496	0	0	0
	MC3	0	0	0	0
	MC4	15.66	0	0	0
	MC5	118	6.18	0.16	0
	MS1	47.6	1.84	5	0
	MS2	9.26	0	9.4	0
	MS3	7.7	0	5.4	0
	MS4	0	0	0	0

Table 4: LC-MS quantification of aflatoxins in clinical and non-clinical isolates of *Aspergillus flavus.*

reported to be involved in removal of excess carbon when the fungi grow on sources rich in carbon [26]. Another possibility is its contribution in processes of fungal growth and development [27]. This phenomenon was also observed in our experiments when fungi were being grown on PDA plates. We observed that *A. flavus* isolates that produced higher levels of toxin grew more aggressively (data not shown) and studies are in progress to address this issue. Other reasons for aflatoxin production consist of its role in facilitating interactions between different species by acting as a chemical signals [28]. According to Drummond and Pinnock [29], aflatoxins may play a protective role against other soil organisms or even insect competitors. Another suggested role is that the fungus makes aflatoxin to counteract the reactive oxygen species produced by plants during droughts or high temperatures [30].

The functional assays in our study exhibited increased cytotoxic effects in case of methanol extract as compared to conditioned media. This is likely because the concentration of aflatoxin in the conditioned media was too dilute so that effects from aflatoxin became negligible. In case of methanol extract, strains producing maximum amount of aflatoxin like C5 and C4, exhibited most cytotoxicity whereas, the strain C3 and S4, that had negligible amount of aflatoxin, exhibited minimal cytotoxic effects (Figure 3A). It is noteworthy that higher cytotoxicity was observed in primary HBMEC, as compared to cancer Huh7 cells (Figure 3A and 3B), likely due to increased resistance of Huh7 cells to AFB1.

Overall, the data suggests that both clinical and non-clinical isolates of *A. flavus* exhibited aflatoxin production, albeit some clinical isolates (C4, C5) produced excessive AFB1 which may be associated with their inherent capacity or external stimilu from the conditions of the environment. Furthermore, strains producing higher amount of AFB1 exhibited increased host cell cytotoxicity, whereas strains exhibiting negligible amount of aflatoxin exhibited minimal cytotoxic effects. Thus the production of AFB1 appears to be a marker for the pathogenic potential of *A. flavus*, however this needs to be tested in future studies involving a large pool of clinical and non-clinical isolates. The ability of aflatoxigenic *A. flavus* to produce host cell death in primary cells raises additional concern for patients suffering from *A. flavus* infection.

Acknowledgements

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