Detection of Gliotoxin as virulence factor in *Aspergillus fumigatus*

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Abstract
Aspergillosis is considered one of the dangerous systemic fungal infections which develops mainly in immunocompromised individuals. The most common cause is Aspergillus fumigatus. Forty-one of A. fumigatus were obtained from a total of 72 sputum specimens; 41 were positive for A. fumigatus, the age of patients ranged between 44 to 77 years old of both gender during September 2011 to March 2012 from chest and respiratory diseases specialized center, Ministry of Health, Baghdad. Aspergillus fumigatus the most causative agent of aspergillosis, the screening the ability of 41 A. fumigatus isolates gliotoxin, melanin, protease and hemolysin production were investigated.

Results indicated that the isolate AFU1 was the highest producer for these four compounds as a virulence factor. In present study there is significant difference in the ability of the 41 A. fumigatus isolates to produce the virulence factors. Gliotoxin and melanin were purified where the optimum gliotoxin production was in rice medium range between (2.8-5.3 µg /gm) and maximum production of melanin ranged between (1.9-3.2 mg/ml).

Keywords: *A. fumigatus*, Gliotoxin, HPLC.

Introduction

Several toxins produced by *A. fumigatus* conidia or hyphae such as gliotoxin have been examined as potential virulence factors.[1] However, to date, most mutants created by deleting the genes encoding enzymes required for the synthesis of specific toxins have demonstrated no reduction in virulence.[2] In contrast, deletion of a gene encoding an enzyme which is required for the biosynthesis of Gliotoxin, displayed reduced virulence.[3]

Gliotoxin C13H14N2O4S2 with a molecular weight 326.4 g/mol has been extensively studied and is considered to be involved in virulence as it inhibits phagocytosis[4] and stimulates apoptosis in macrophages[5] and therefore has been demonstrated to possess immunosuppressive properties. The role of gliotoxin in the virulence of *Aspergillus* species in different host models has been reviewed recently.[6] Gliotoxin is an epipolythiodioxopiperazine (ETP).

One of the most likely virulence determinants among various secondary metabolites produced by the species *A. fumigatus* is the most frequent cause of invasive Aspergillosis worldwide and over 90% of the strains isolated from invasive Aspergillosis cases in tertiary care cancer centers were found to be gliotoxin producers.[8] Furthermore, the amount of toxin produced by *A. fumigatus* was substantially higher than the amount produced by other less frequent species of pathogenic *Aspergillus* including *A. terreus* and *A. flavus*.[9]

Gliotoxin inhibits the phagocytosis by macrophages and can induce their apoptosis.[10] Gliotoxin would play an important role in the pathogenesis of *A. fumigatus* in these patients.[11] Since gliotoxin is also an ETP toxin, they proposed it to be the putative gliotoxin biosynthetic gene cluster. Similar ETP types of gene clusters were subsequently found in many other fungi belonging to Ascomycetes.[12] The discovery of the putative gliotoxin biosynthetic gene cluster in *A. fumigatus* allowed us to determine the toxin playing any role in the pathobiology of *A. fumigates*.[13]

Material and Methods

Samples collection: Sputum samples were collected from 72 patients (44 to 77 years old of both sexes 44 male, 28 female) suspected have infection with aspergillosis (as clinically identified by a physician), during the period of September/2011 to March/2012 from chest and respiratory diseases specialized center, Ministry of Health, Baghdad Province. The samples were examined directly under the microscope using 10% KOH and culturing on the sabouraud dextrose agar.[14]

Samples Culturing: Sputum samples were cultured on sabouraud dextrose agar (SDA) supplemented with 0.05 mg/ml chloramphenicol to inhibit the growth of bacteria and then incubated at 25°C and 37°C for 10 days.[15]

Identification of Isolates: All isolates were identified to the species level on the basis of macroscopic and microscopic characteristics using SDA and Scotch tape preparation. A small piece of transparent-adhered tape was touched to the surface of the suspected colony and then adhered to the surface of a microscope slide to which a drop of lactophenol cotton blue was added. Shape and arrangement of the spores were examined microscopically.[16] The isolates were identified according to Nieminen et al.[17]

Preparation of spore suspension for *Aspergillus fumigatus* isolates: After the isolates were cultured on sabouraud dextrose agar at 25 °C for 7 days, fungal colonies were covered with 10 ml of sterile saline solution for spore suspension preparing and the suspensions were prepared by gently agitation of the surface with the tip of a pasture pipette. The spore suspension was filtered through sterile gauze and then the filtration was transferred to a sterile test tube. Inoculums quantification was made by counting the spores using haemocytometer by adding one drop of the suspension to haemocytometer by Pasteur pipette; spores were calculated under high power 40X of light microscope using the following equation:

Concentration of spores = (Z *4 * 10^6) /n spores/ml

where n = total no. of small squares and Z = total no. of spores.[18]

Screening of *A. fumigatus* isolates for gliotoxin production: Gliotoxin production on rice medium was achieved according to Sambrook et al[22] with modification Each of 41 isolates of *A. fumigatus* was cultured on rice medium triplicate for each isolate. Cultures of each media were inoculated with 5mm of cork borer from one-week old sabouraud dextrose agar culture of each tested isolates. Inoculated rice cultures were incubated for 7 days at 25°C. Gliotoxin was extracted from rice medium (each flask) two time by shaking 8-12 hr with 250 and 200 ml of chloroform respectively.

The extracts were combined, filtered evaporated and re-dissolved in 10ml chloroform before 250 petroleum ether was added and the mixture was placed at 4°C overnight; the solvent was evaporated at 50°C, the residue re-dissolved in 5 ml methylene chloride: cyclohexane (50:50v: v).

The solvents in each fraction were evaporated to dryness, each fraction residue was transferred quantitatively with methylene chloride to a vial and solvent evaporated at 50°C, the residue in each vial was dissolved in 5ml of methanol: water (43:57 v:v) and stored at -70°C until HPLC analysis.

Gliotoxin production on Czapex-dox broth medium: For gliotoxin production, each of 41 *A. fumigatus* isolates cultured on sabouraud agar plates for 2 days at 25°C and conidia harvested using sterile Tween 20. Concentration of conidia was adjusted to $10^7 ml^{-1}$ in distilled water using haemocytometer according to Rafael et al[19] by following steps:

- 1ml of each conidial suspension used to inoculate 100 ml of czapex-dox broth in a 500 ml flask.
- Culture incubated at 25°C in a shaking incubator at 1400 rpm for 2 days.
- Fungal biomass harvested by filtration through Whatmann no. 1 filter paper in Buchner funnel.
- The filtrate extracted three times by shaking for 10 min with 50ml chloroform at 25°C.
- Separate the chloroform fractions and evaporate to dryness on a rotary evaporator at reduced pressure and at 47°C.
- Dried extracts dissolved in 200µl methanol and store at -70°C until HPLC analysis.

High performance liquid chromatography (HPLC) analysis: For HPLC analysis, the toxin samples were diluted 1:10 and 20µl of each fraction was injected. The HPLC system considered of a 10 cm x4.6mm RP analytical column (10 micrometer) with a 3 cm guard column with 10µl RP - 18 packing, a water model 510 pmp equipped with a U6K septum-less injector with a 254 nm filter linked to a Shimadzu CRI-B data processor. The mobile phase was methanol: water (43:57) and flow rate was 2 ml min. Injection of 45, 90 and 180 ng of standard gliotoxin dissolved in the mobile phase was conducted to determine retention time RT and relative peak area. A standard curve of the relationship of peak area quantity (ng) injected was constructed and used for sample concentration of gliotoxin according to the formula.[20] Statistical analysis has been applied for all concentrations and the control mean ± standard error and differences between means was ascertained by Anova.[21]

Results and Discussion

Isolation and Identification of *Aspergillus fumigatus* from Clinical samples: A total of 72 sputum samples from suspected patient with Aspergillosis, were examined by 10% KOH. Cultural and microscopic examination illustrates that the infections belonged to the fungus *Aspergillus fumigatus* in 41 sputum.[22]

Direct KOH examination illustrates septet hyphae and dichotomous branching, conidial heads produced in chains basipetally from phialides, chains of conidia are borne directly on broadly clavate vesicles in the absence of metulae. Using potassium hydroxide alkaline solutions, the fungus remains unaffected.[23]

Upon culturing on sabauroud dextrose agar supplemented with chloramphenicol (SDA), colonies of *A. fumigatus* appear fast grower; the colony size can reach 7 cm within a week when grown on SDA and 4 cm when grown on Czapek-Dox agar at 25°C , the colony powdery, the color at the first seems to be white, then turning to dark greenish and changing to gray, reversed side of the colonies appeared pale yellow to tan. Figure 2 shows the colony of *A. fumigatus*.

Microscopic examination as shown in figure 3 appeared clavate vesicles conidia, 2.5 to 3 µm in diameter, phialides arrange uniseriate upper vesicle conidia and parallel to axis of conidophore, produced in chains of spore basipetally from phialides, the chains of spore are borne directly in the absence of metulae and represented by septet and branching hyphae.[23]

Ability of *A. fumigatus* isolates to grow at 55°C and 70°C: One of the diagnostic test for *A. fumigatus* is the ability to grow at abroad range of temperature. Forty-one isolates of *A. fumigatus* examined showed its ability to grow at 55°C and 70°C on SDAC. The result indicated that all the examined isolates had the ability to grow at temperatures 55°C and maintained survival at 70°C. The average diameter of growth colony ranged between (4-7cm) with spread growth at first, this result agrees with all the studies regarding diagnosis test of *A. fumigatus*.[24]

Analysis of case reports from patients with Aspergillosis: The significance of Aspergillosis has dramatically increased with growing numbers of patients with impaired immune state associated with the management of malignancy, organ transplantation, autoimmune and inflammatory conditions; critically ill patients and those with chronic obstructive pulmonary disease appear to be at an increased risk, *A. fumigatus* which may lead to a variety of infectious, depending on the host's immune status aspergillosis occurs primarily in patients with severe immunodeficiency.[21]

Table 1 summarizes all information obtained from each patient, cultural and microscopic examination confirms the presence of *A. fumigatus* as the main causative agent of aspergillosis in the present study and this result agrees with the result of studies.[12] These *A. fumigatus* isolates included in this study isolated from patients came to specialized center.

The infection with *A. fumigatus* in male patient 29 (70.3%) was higher than female 12(29.3%). This result partially agreed with the result of Schnabl et al[24] who found that aspergillosis in male was almost twice that at female patients a ratio of 20: 10. Aspergillosis infection was more prevalent in age between (44-47) years old; the mean age of the patients was 58±8.29 years old ,the age range was broad, this result agrees with the result of Sutton et al[25] who reported the aspergillosis in the patient old more than 50 years old.

In case of Aspergillosis infection associated with disease, the results in table 1 revealed that from 41 patients, 15 (36.5%) patients with aspergillosis had different cancer infections and the same percentage 15 (36.5%) patients had tuberculosis while 11 (27%) infected with diabetes. In this study each patient had a disease that was complicated by aspergillosis, 15 patients with cancer were suffering from Leukemia. One patient had carcinoma of the breast, another patient had carcinoma of the stomach and one had carcinoma of the bladder in addition to leukemia.

The occurrence of aspergillosis in patients with pulmonary tuberculosis has been reported in this study. Pulmonary tuberculosis is one of the most common chronic pulmonary infections caused by *Mycobacterium tuberculosis* in patients with human immunodeficiency, virus infection and those requiring immunosuppressive therapies are more susceptible to tuberculosis with aspergillosis.[22] Association of diabetes disease with Aspergillosis in 27% from cases in this study reported the uncontrolled diabetes and advanced age along with the invasion of the lung.[7] Three forms reported in this study, 30 (73.2%) allergic bronchopulmonary Aspergillosis (ABPA), 6 (14.6%) Aspergilloma and 5 (12.2%) invasive Aspergillosis IA.

Pulmonary diseases caused by *A. fumigatus* can be classified according to the site of the disease within the respiratory tract and the extent of mycelia colonization or invasion, both of which are influenced by the immunological status of the host.[9] Exposure to conidia or antigens of *A. fumigatus* in the absence of mycelial colonization and in most cases, removal of the patient from the environmental source results in clinical improvement. In contrast, allergic bronchopulmonary Aspergillosis (ABPA) is the most common form of Aspergillosis, Aspergilloma and IA, syndromes involving mycelia growth of *A. fumigatus* in the body, usually requiring therapeutic intervention. Classification of Aspergillosis of respiratory Aspergillosis depends on clinical symptoms and diagnosis in this study. This result of classification partially agreed with the result of Wang et al.[26]

Screening of *A. fumigatus* isolates for gliotoxin production in Rice medium and Czapek-Dox broth medium: The ability of *A. fumigatus* isolates for gliotoxin production was determined using rice medium as a solid-state fermentation and Czapek-Dox broth medium as a submerged culture, the occurrence of gliotoxin in 41 *A. fumigatus* isolates was detected by High Performance Liquid Chromatography (HPLC). High Performance Liquid Chromatography (HPLC) is an important qualitative and quantitative technique, generally used for the estimation of pharmaceutical and biological samples. It is the most versatile, safest, dependable and fast test chromatographic technique for the quality control of components.[27]

The results in table 2 showed that high gliotoxin production on rice medium, 35(85.36%) isolates gave positive result by HPLC, significant difference were shown $P<0.001$ between the gliotoxin producers isolates, concentration of gliotoxin in rice medium ranged between 0.2 – 5.3 µg/gm. The isolates AFU1, AFU15, AFU23, AFU30 and AFU40 gave the highest concentration ranged between 2.8-5.3 µg/gm rice medium for highest production of gliotoxin from environmental *A. fumigatus* isolates.

Solid state fermentation SSF is generally insoluble in water. In practice, water is absorbed onto the substrate particles, which can then be used by organisms for growth and metabolic activities. Depending upon the nature of the substrate, the amount of water absorbed could be one or several times more than its dry weight, which leads relatively to high water activity on the solid / gas interface in order to allow higher rate of biochemical process.[28] While in Czapek-Dox broth medium the results showed that 27(65.85%) isolates gave positive result by HPLC analysis, significant

difference were shown ($P < 0.001$) between the gliotoxin producers isolates, concentration of gliotoxin in Czapek-Dox broth medium ranged between (1.1–2.3µg/ml). Maximum yield of gliotoxin were obtained from rice culture medium as indicated by HPLC analysis when compared with yield in Czapek-Dox broth medium as shown in table 2. The HPLC solvent used for the separation of gliotoxin from other compounds in the extract of rice cultures was methanol /water (43:57v/v), as described for HPLC determination.[28] This was the only solvent system that was tried and was very satisfactory and represented the retention time for gliotoxin about 4.8 min (fig. 4 and 5).

There were no peaks evident at retention times immediately adjacent to that of gliotoxin. The HPLC procedure was applied to gliotoxin spiked samples of rice that had been prepared similarly to those used for cultures of *A. fumigatus.* The percent of recovery was based on HPLC peak area compared to that of standard gliotoxin. No other fraction contained detectable gliotoxin by HPLC. There was linear relationship of peak area of the recovered amount of gliotoxin from spiked samples with the concentrations of the gliotoxin in the rice from (1.2-5.3µg /gm).

This study indicated that Rice medium is a better medium for screening large numbers of *A. fumugatus* isolates for gliotoxin production which is both quick, easy, cheap and available.

It is possible to increase mycotoxin production and using solid state fermentation (SSF), therefore it is important to adjust production mechanisms of secondary metabolites and optimization of nutrients for various strains for better mycotoxin production.[29] The species. *A. fumigatus* is the most frequent cause of invasive Aspergillosis worldwide and over 90% of the strains isolated from invasive Aspergillosis cases in tertiary-care cancer centers were found to be gliotoxin producers.[30] Furthermore, the amount of gliotoxin produced by *A. fumigatus* was various between the strains.[28] Although all the isolates in this study were pathogenic but 14.6% of the isolate did not produce gliotoxin in rice medium and 34.1% from the isolate did not produce in Czapek-Dox broth medium, not every strain is a gliotoxin producer.

These results agreed with several reports that cite the frequency of toxin producing strains that have been isolated from the environment versus clinical specimens from patients. Gardiner et al[12] reported that only 11% of the *A. fumigatus* strains isolated from patient in the Azores were gliotoxin producers. In contrast, 93% of the *A. fumigatus* strains (n =40) were isolated between 1998 and 2003 from respiratory and tissue samples of cancer patients. Gliotoxin producers compared to 75% and 25% of *A. fumigatus* (n =9) (n =27) strains, respectively were isolated from patients with Aspergillosis.

$$GT = \frac{PASP}{VST} * \frac{VST}{S} * \frac{S}{W}$$

$$GT = \frac{PASP}{VSP} * \frac{VSP}{W}$$

GT= concentration of gliotoxin, ng/g;

PASP= peak area standard gliotoxin(count);

VST=volume standard injected µl,

Figure 1: Relation of GT, PASP and VST

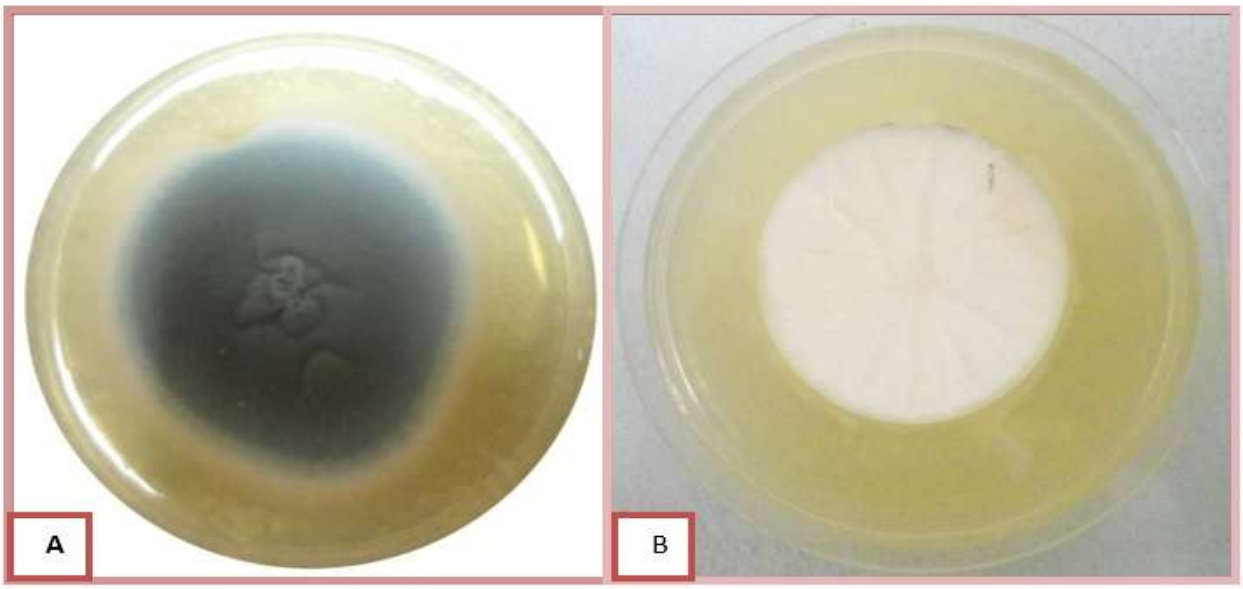

Figure 2: *Aspergillus fumigatus* grown on SDAC at 37°C after 7 days of incubation. A- Top view B- Reversed view

Figure 3: Microscopic feature of *Aspergillus fumigatus* stained with Lactophenol cotton blue(40x) .

Furthermore, the concentrations of gliotoxin produced by *A. fumigatus* isolated from patients were significantly higher than those of other environmental isolates.[17,30,31] Very different frequencies of gliotoxin producing *A. fumigatus* strains were recovered from immunocompromised patients with various diseases in the hematology unit at the University of Zagreb in Croatia. Out of the 50 clinical isolates, only 18% produced gliotoxin while no environmental strains of an equivalent number screened were found to produce the toxin.

Although the data are fragmentary, the results suggest that the frequency of gliotoxin producing *A. fumigatus* strains varies depending on the geographic region and/or type of patients that yielded the fungal strains. It appears, however, that the frequency of finding gliotoxin producing *A. fumigatus* strains is higher among clinical isolates than among environmental isolates.[29,32]

Table 1
Analysis of case reports from 41 patients with aspergillosis.

S.N.	Location	Sex	Age	Associated diseases	Direct Examination	Cultural Examination	Classification of Aspergillosis	Symbol of isolate
1	Baghdad	F	63	diabetes	+	*A. fumigatus*	ABPA	AFU1
2	Salahaldeen	M	65	diabetes	+	*A. fumigatus*	ABPA	AFU2
3	Baghdad	M	59	diabetes	+	*A. fumigatus*	ABPA	AFU3
4	Baghdad	M	44	diabetes	+	*A. fumigatus*	ABPA	AFU4
5	Baghdad	M	49	diabetes	+	*A. fumigatus*	ABPA	AFU5
6	Baghdad	M	52	diabetes	+	*A. fumigatus*	ABPA	AFU6
7	Baghdad	M	77	Cancer	+	*A. fumigatus*	**aspergilloma**	AFU7
8	Dyala	M	61	Cancer	+	*A. fumigatus*	IA	AFU8
9	Dyala	M	75	Cancer	+	*A. fumigatus*	ABPA	AFU9
10	Baghdad	M	74	Cancer	+	*A. fumigatus*	IA	AFU10
11	Baghdad	F	58	Cancer	+	*A. fumigatus*	ABPA	AFU11
12	Baghdad	F	50	diabetes	+	*A. fumigatus*	ABPA	AFU12
13	Kerbalaa	M	54	Cancer	+	*A. fumigatus*	ABPA	AFU13
14	Baghdad	M	60	diabetes	+	*A. fumigatus*	ABPA	AFU14
15	Baghdad	M	67	tuberculosis	+	*A. fumigatus*	ABPA	AFU15
16	Sulaimania	M	63	tuberculosis	+	*A. fumigatus*	ABPA	AFU16
17	Baghdad	M	54	Cancer	+	*A. fumigatus*	ABPA	AFU17
18	Baghdad	M	66	tuberculosis	+	*A. fumigatus*	**aspergilloma**	AFU18
19	Baghdad	M	68	Cancer	+	*A. fumigatus*	IA	AFU19
20	Baghdad	M	64	diabetes	+	*A. fumigatus*	ABPA	AFU20
21	Baghdad	M	49	tuberculosis	+	*A. fumigatus*	ABPA	AFU21
22	Baghdad	M	52	diabetes	+	*A. fumigatus*	ABPA	AFU22
23	Baghdad	F	71	diabetes	+	*A. fumigatus*	ABPA	AFU23
24	Baghdad	F	63	tuberculosis	+	*A. fumigatus*	ABPA	AFU24
25	Baghdad	F	69	tuberculosis	+	*A. fumigatus*	ABPA	AFU25
26	Baghdad	F	52	Cancer	+	*A. fumigatus*	IA	AFU26
27	Baghdad	F	66	tuberculosis	+	*A. fumigatus*	ABPA	AFU26
28	Najaf	M	47	tuberculosis	+	*A. fumigatus*	ABPA	AFU28
29	Baghdad	M	55	Cancer	+	*A. fumigatus*	ABPA	AFU29
30	Baghdad	M	59	tuberculosis	+	*A. fumigatus*	**aspergilloma**	AFU30
31	Kerbalaa	M	52	tuberculosis	+	*A. fumigatus*	ABPA	AFU31
32	Baghdad	M	45	tuberculosis	+	*A. fumigatus*	ABPA	AFU32
33	Baghdad	M	51	tuberculosis	+	*A. fumigatus*	ABPA	AFU33
34	Najaf	F	60	tuberculosis	+	*A. fumigatus*	aspergilloma	AFU34
35	Baghdad	F	49	tuberculosis	+	*A. fumigatus*	**aspergilloma**	AFU35
36	Wasit	M	62	tuberculosis	+	*A. fumigatus*	ABPA	AFU36
37	Basrah	F	70	cancer	+	*A. fumigatus*	IA	AFU37
38	Baghdad	F	47	cancer	+	*A. fumigatus*	ABPA	AFU38
39	Baghdad	M	44	cancer	+	*A. fumigatus*	**aspergilloma**	AFU39
40	Baghdad	M	65	Cancer	-	*A. fumigatus*	ABPA	AFU40
41	Baghdad	M	73	Cancer	-	*A. fumigatus*	ABPA	AFU41

+ (Positive result) = growth appearance, M= male, F= female, IA= invasive Aspergillosis, ABPA= allergic bronchopulmonary Aspergillosis.

Table 2
Ability of *Aspergillus fumigatus* isolates in production of gliotoxin in Rice and Czapek-Dox broth medium.

S.N.	**Isolates**	**Gliotoxin concentration µg/gm on Rice medium mean ±SE 7 days at 28°C**	**Gliotoxin concentration µg/ml on Czapek-Dox broth media mean ±SE, 48 Hrs, 37°C**
1	AFU1	5.3±0	2.3± 0
2	AFU2	2.1±0	1.7±0.3
3	AFU3	1.9±0	1.1±0.3
4	AFU4	1.2±0	0±0
5	AFU5	0.7±0.1	0 ±0
6	AFU6	0.5±0.2	0±0
7	AFU7	1.0±0	1.7±0.05
8	AFU8	1.2±0	1.2±0. 5
9	AFU9	1.8 ±0	1.7±0.6
10	AFU10	2.0 ±0	1.9±0.2
11	AFU11	2.0 ±0	1.3±0.3
12	AFU12	2.9 ±0	1.9±0.05
13	AFU13	2.2 ±0	1.5±0.05
14	AFU14	1.5 ±0.1	0 ±0
15	AFU15	2.8 ±0.06	1.1±0.3
16	AFU16	1.8±0.1	0 ±0
17	AFU17	1.8±0.1	0 ±0
18	AFU18	1.2 ±0	1.1±0.3
19	AFU19	0.2 ±0	1.1±0.3
20	AFU20	1.5 ±0.1	0±0
21	AFU21	0 ±0	0 ±0
22	AFU22	0± 0	0 ±0
23	AFU23	3.1±0.1	1.1±0.3
24	AFU24	0±0	0 ±0
25	AFU25	0 ±0	0 ±0
26	AFU26	3.0±0.1	1.6±0.02
27	AFU26	3.0±0.2	1.9±0.05
28	AFU28	2.8±0.1	1.8±0.04
29	AFU29	3.2±0.05	1.1±0.3
30	AFU30	3.8 ±0.1	1.9±0.03
31	AFU31	2.9±0.1	0±0
32	AFU32	3.0 ± 0	1.2±0.3
33	AFU33	1.2 ±0	1.1±0
34	AFU34	3.0 ±0.2	1.9±0.05
35	AFU35	1.9 ± 0.1	1.1±0.3
36	AFU36	1.7 ± 0	1.1±0. 5
37	AFU37	0 ±0	0 ±0
38	AFU38	2.7± 0.1	1.2±0.3
39	AFU39	3.0 ± 0	1.1±0.3
40	AFU40	5.0 ± 0.1	1.1±0.3
41	AFU41	0 ±0	0 ±0

Significant Differences $p<0.001$

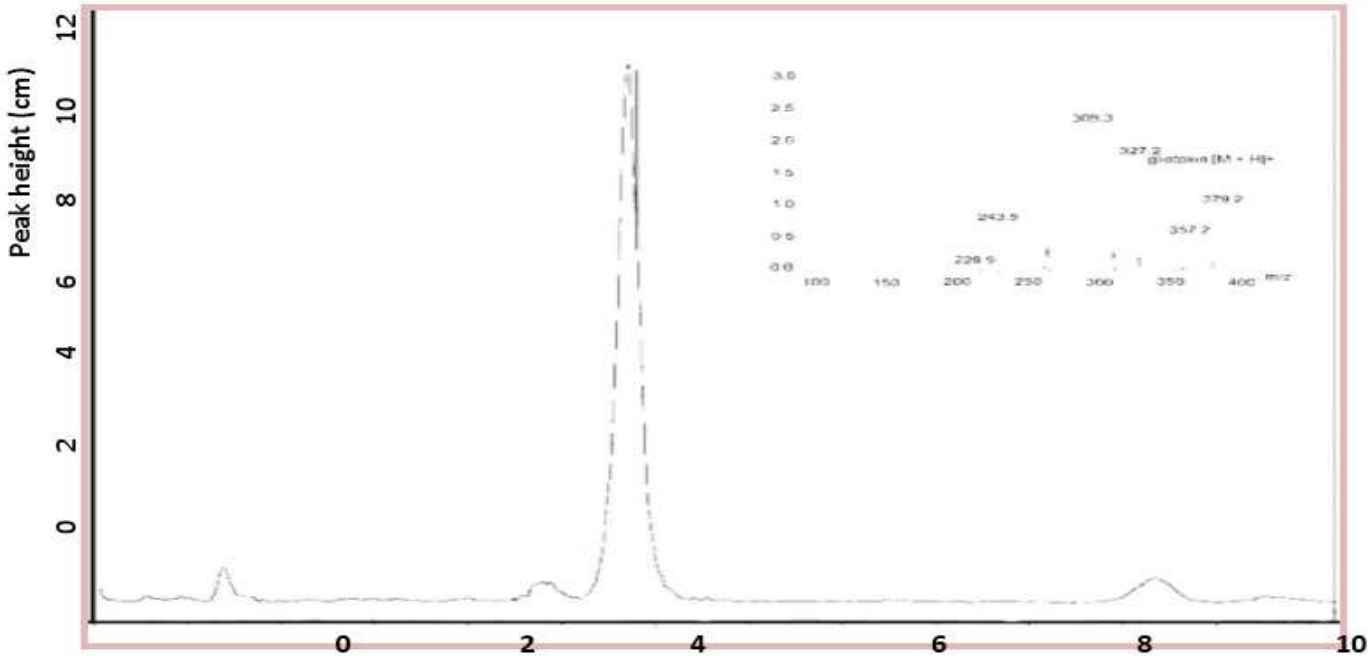

Figure 4: Detection of standard Gliotoxin by HPLC analysis method

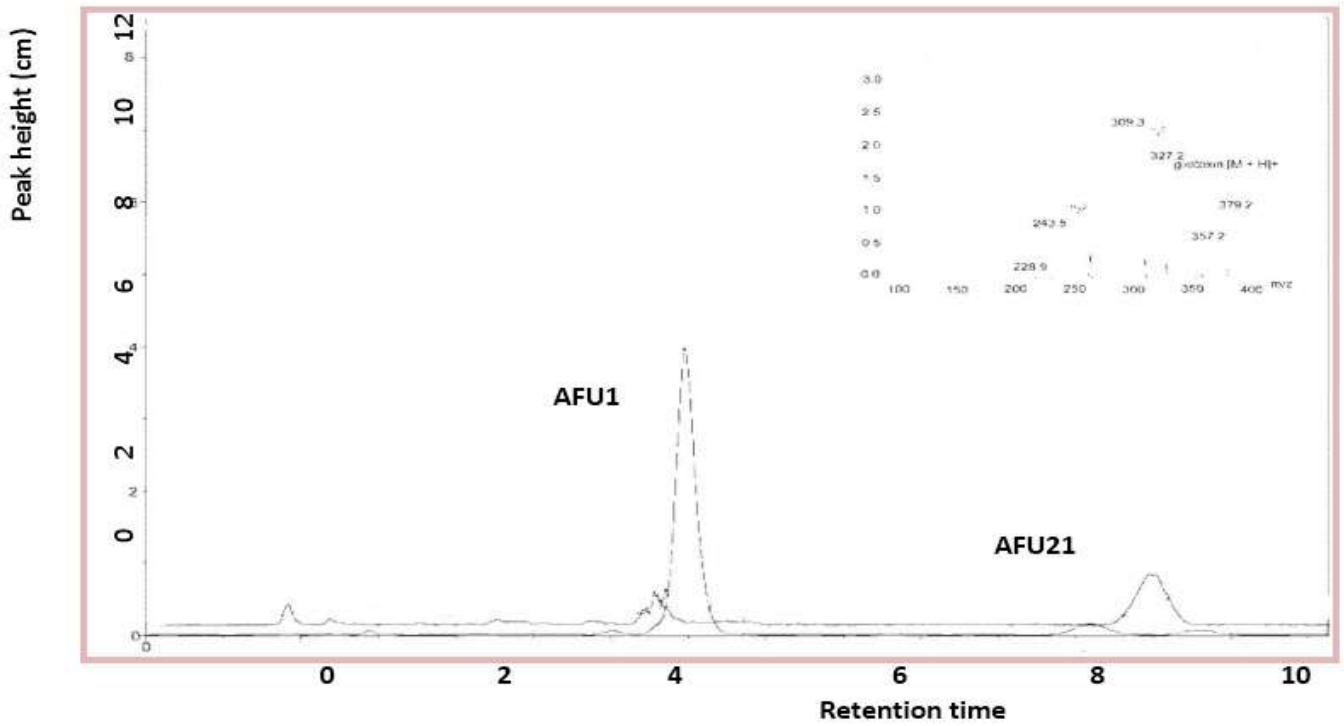

Figure 5: Detection of purified gliotoxin produced by *A. fumigatus* on Rice media conditions using HPLC analysis method, gliotoxin retention time approximately 4.8 min., The isolate AFU1: gliotoxin producers, AFU21: non gliotoxin producers. The mobile phase was methanol: water (43:57), flow rate was 2.0 ml min.

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