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Optimal Conditions for Gliotoxin Production from *Aspergillus fumigatus* Using Solid State Fermentation

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Abstract

The ability of ten *Aspergillus fumigatus* isolates to produce gliotoxin was assessed by solid state fermentation using rice medium as a substrate. The results indicated that the AF-5 *Aspergillus fumigatus* isolate produced the most gliotoxin. The optimum conditions for gliotoxin production were a moisturising ratio of 5:1 (w: v) with distilled water, an inoculum size of 1.5 ml, containing 6×10^6 spores and incubation at 37 ºC for 10 days using rice medium as a substrate. Gliotoxin was partially purified from the crude extract by a two stage process of filtration and solid phase extraction. The purity and concentration of the produced gliotoxin was assessed using Thin layer Chromatography and High Performance Liquid Chromatography. The retention time for gliotoxin was found to be approximately 10.2 min and its concentration was 122.6 ppm.

***Keywords*:** Aspergillus fumigatus; Gliotoxin; optimum conditions; solid state fermentation.

1. Introduction

Invasive aspergillosis in humans is a disease associated with *Aspergillus fumigatus.* As a saprophyte, *Aspergillus fumigatus* produces a range of secondary metabolites, such as mycotoxins, during its invasive growth, and poses a major risk to humans, animals and crops [1,2].

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The epipolythiodioxopiperazine (ETP) metabolite, gliotoxin, is one of the most common of these metabolites and is considered likely to be a significant virulence factor. Gliotoxin is a first epipolythiodioxopiperazines (ETP's) reported and is the best characterized (Fig. 1). Its name was derived from its identification as a metabolite of *Gliocladium fimbriatum* first isolated from cultures of the fungus *Gliocladium*. Gliotoxin is implicated in animal mycoses. It is immunosuppressive and causes apoptotic and necrotic cell death in vitro. The toxicity of ETP's led to use it as a therapeutic agent for diseases such as cancer [3,4]. Gliotoxin ($C_{13}H_{14}N_2O_4S_2$) has a molecular weight of 326.4 g/mol, and has been extensively studied since it inhibits phagocytosis (Eichner and his colleagues 1986) and stimulates apoptosis in macrophages (Orciuolo and his colleagues 2007).Gliotoxin plays an important role in the pathogenesis of aspergillosis in patients immunosuppressed [5].

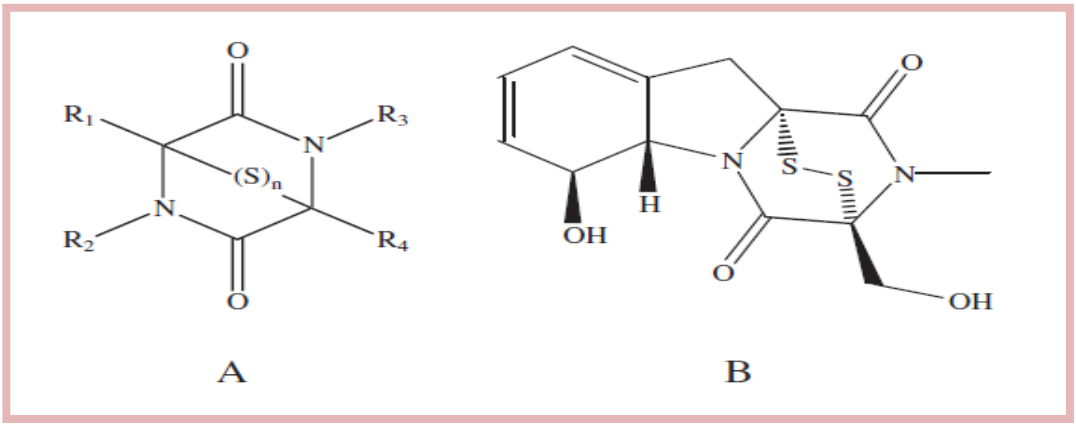

Figure 1: (A) Genericstructure ofepipolythiodioxopiperazines. **(B)** Structureof gliotoxin (Anitha and Murugesan, 2005).

The objective of the present study was to identify the optimum conditions for the production of gliotoxin from local *Aspergillus fumigatus* isolates using solid state fermentation (SSF), and then to determine its purity and concentration using Thin Layer Chromatography (TLC) and High Performance Liquid Chromatography (HPLC).

2. Methods

2.1 Fungal Isolates

Ten pure cultures of *Aspergillus fumigatus* isolates were obtained from the Department of Biotechnology, College of Science, University of Baghdad. Fungal isolates were maintained in PDA slant medium and were kept at 4 °C as stock cultures, with a subculture being done every three months [6].

2.2 Preparation of Rice, Wheat, Barley and Corn media

Twenty five grams of long-grain rice, wheat, barley and corn were placed in separate 250 ml cotton-stoppered Erlenmeyer flasks, sterilized by autoclaving at 121 ºC, 15 Psi for 15 min three times over a period of three days and then each used as a solid substrate for gliotoxin production. The moisturising ratio was 5:1 (w/v) by D.W **[7]**.

2.3 Screening the ability of Aspergillus fumigatus isolates to produce gliotoxin

Richard and his colleagues [8] achieved gliotoxin production on a rice medium. Following this method, each of the ten isolates of *A. fumigatus* were cultured on a rice medium, in duplicate for each isolate. The media were inoculated with 1 ml of spore suspension of each of the tested isolates. Inoculated rice cultures were incubated for 7 days at 28 °C.

2.4 Extraction of gliotoxin

Following the incubation period, the rice in each flask was extracted twice with 75 ml and 50 ml of chloroform, respectively, using an electric homogenizer for 10 min. The extracts were combined, filtered through a Whatman No.1 filter paper and evaporated to dryness at 50 °C. The dried extracts were stored at 4 °C until TLC analysis [9].

2.5 Detection and Quantification of the gliotoxin

- **Thin layer chromatography (TLC) analysis**

The TLC plates were activated in an oven at 120 °C for 1 hour before use [10]. The dried extracts, prepared as described above, were now redissolved in 2 ml ethyl acetate. 20 µl of each of the dissolved extracts was place on a silica gel 60 plate, which was then developed in a TLC tank with a methylene chloride: methanol (97:3 v/v) solvent system as the mobile phase. The plate was then air-dried before being sprayed with a reagent of 5% ethanolic silver nitrate: water (90: 5 v/v). The TLC plates were used to screen for the presence of fluorescent compounds by viewing them under a UV box at a wavelength of 365 nm [8].

- **High Performance Liquid Chromatography (HPLC) analysis** [8].

The conditions for the HPLC analysis were as follows:

- ❖ Instrument: Sykam- Germany
- ❖ Analytical column: C18 (30 cm x4.6 mm)
- ❖ Loop: 50 µl
- ❖ Wavelength: Det- UV- 254 nm
- ❖ The mobile phase: methanol: water (43:57)
- ❖ Flow rate: 2 ml/min.
- ❖ Concentration of standard gliotoxin: 1000 ppm

Twenty µl of both standard gliotoxin and sample gliotoxin were assessed through HPLC to determine the retention time (RT) and relative peak area. The concentration of the sample gliotoxin was calculated according to the following formula.

$$\mathbf{C}_{st.} = \frac{\mathbf{C}_{s.}}{\mathbf{A}_{st.}\mathbf{A}_{s.}}$$

$\mathbf{C}_{st.}$= concentration of standard gliotoxin (ppm)

$\mathbf{A}_{st.}$= peak area standard gliotoxin (count)

$\mathbf{C}_{s.}$= concentration of sample gliotoxin (ppm)

$\mathbf{A}_{s.}$= peak area sample gliotoxin (count)

2.6 The effect of different conditions on gliotoxin production

Culture media Selected isolates of *A. fumigatus* were grown in each of four different media (corn, barley, wheat and rice) with each medium being inoculated with 1 ml of spore suspension and then incubated at 28 °C for 7 days.

Incubation times Twelve flasks of rice medium duplicate cultures of each isolate were inoculated with 1 ml of spore suspension before being incubated at 28 °C for different incubation times of 3, 5, 7, 10, 12 and 14 days.

Moisturising ratio Ten flasks of rice medium duplicate cultures of each isolate were inoculated with 1 ml of spore suspension under different moisturising ratios (5 : 0.5 , 5 :1 , 5 :1.5 , 5 :2 , and 5 :2.5) (w/v). All the flasks were incubated at 28 °C for 10 days.

Temperatures Eight flasks of rice duplicate cultures of each isolate were inoculated with 1 ml of spore suspension with all of the flasks being incubated atdifferent temperature (28, 32, 37 and 42° C) for 10 days.

Inoculum size Ten flasks of rice medium duplicate cultures of each isolate were inoculated with differentinoculum sizes of 0.25, 0.5, 1, 1.5 and 2 ml of spore suspension with the volume being made up to 5 ml with distilled water. The flasks were incubated at 37 °C for 10 days. Inoculum size was calculated according to the following equation:

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

Where n: the average spore count / squares [9]

After determine optimum condition the toxin was extracted with chloroform and stored in a small vial at 4 ºC until analysed by TLC and HPLC.

2.7 Purification of gliotoxin

The first stage of the purification entailed combining, evaporating and redissolving all of the extracts in 2 ml methylene chloride. They were then filtered through 0.45 µm millipore filters and placed on a silica gel Sep-Pak column. The extracts were then primed with 5 ml of methylene chloride and the Sep-Pak columns were eluted with 2 ml of each of hexane, ethyl acetate, chloroform, and methanol. Each eluate was evaporated until it was dry and then redissolved in 1 ml methylene chloride to enable detection. The concentration of the toxin concentration was determined by TLC and HPLC and compared with the specific gliotoxin standard provided by Sigma-Aldrich [8].

3. Results and discussion:

3.1 Screening of A. fumigatus isolates in a rice medium for their production of gliotoxin

The ability of ten *A. fumigatus* isolates to produce gliotoxin was determined using rice medium as a solid state fermentation at 28 °C after 7 days of incubation. The chromatographic analysis using TLC indicated that only oneisolate, AF-5, had the ability to produce gliotoxin (Fig. 2). The efficiency of fungal isolates in producing mycotoxins may be affected by both environmental and genetic factors. [10], for example, reported that only 11% of the *A. fumigatus* strains isolated from patients in the Azores were gliotoxin producers. Kosalec and Pepeljnjak [11], meanwhile, reported that different *A. fumigatus* strains possessed very different gliotoxin production abilities. Out of 50 clinical isolates in their study, only 18% produced gliotoxin. They argued that this suggests that whether particular *A. fumigatus* strains can produce gliotoxin depends on the geographic region and/or type of patients that yielded the fungal strains. It appears, however, that there is a greater likelihood of finding gliotoxin producing *A. fumigatus* strains among clinical isolates than among environmental isolates [12]. Furthermore, the amount of gliotoxin produced by *A. fumigatus* varies between strains [13,14]. The separation of compounds in TLC is based on the competition of the solute and the mobile phase for binding places on the stationary phase. The separation of components is measured by the retardation factor (R_f) value or by using fluorescent reagents that allow the visualization of spots under UV-Cabinet light. Before being sprayed with the silver nitratereagent, gliotoxin on TLC plates appeared as a brown colour (in visible light) and as a yellow colour under UV at 365 nm. After spraying, however, although it still appeared brown in visible light it was dark brown under UV at 365 nm. Similar results were obtained by [15].

Figure (2): Detection of the ability of ten *A. fumigatus* isolates to produce gliotoxin on a rice medium. Using TLC analysis, the mobile phase was methylene chloride: methanol (97:3), the spray reagent 5% silver nitrate and gliotoxin appeared as a brown colour.**No.1 to10:***A. fumigatus* isolates**, St:** Standard gliotoxin, **R:** Rice medium only.

3.2 Optimum conditions for gliotoxin production by solid state fermentation:

Effect of culture media

The *A. fumigatus* isolate AF-5 could be grown on all culture media used to produce gliotoxin, but the substrates varied in their ability to induce gliotoxin production. In rice and barley media the growth of the isolate was better than in corn and wheat media (Fig. 3), indeed the extent of fungal growth was almost identical in rice and barley media. Chromatographic analysis using TLC indicated, however, that while the rice medium gave good production of gliotoxin there was no gliotoxin production with any of the other culture media (Fig. 4). Overall, therefore, a rice medium is the best culture medium for gliotoxin production from *A. fumigatus* isolates. And also has the advantage of being quick, easy, cheap and available.The fact that rice is a more efficient producer than barley may be because barley contains less carbohydrate (62.7 %) compared to rice (73.7 %), in addition, barley contains a higher percentage of fibre (9.7%) compared to rice (2.2 %)the difference may be related to its contents (Belitz and his colleagues 2009).

Figure 3: *A. fumigatus* isolate AF-5 grown on different culture media, moisture ratio 5:1 (w/v), inoculum size 1 ml, incubated at 28 °C for 7 days.

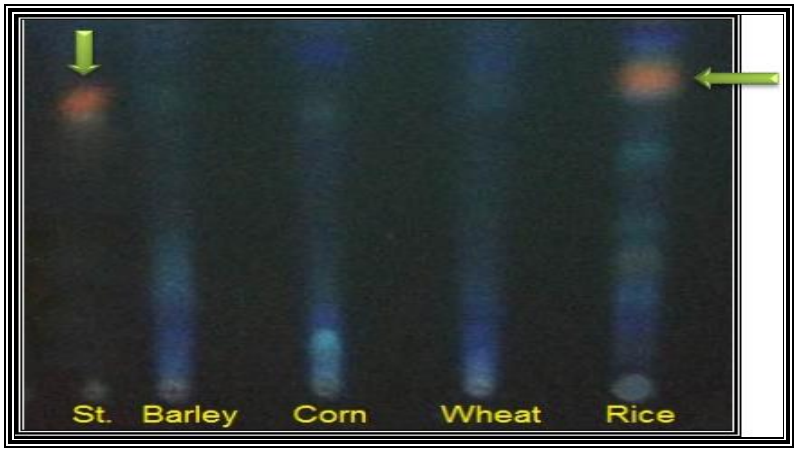

Figure 4: Detection of the ability of *A. fumigatus*AF-5 isolate to produce gliotoxin on different culture media.

Richard and his colleagues (1989) have studied the ability of *A. fumigatus* to produce gliotoxin using Eagle's F-15, YES and rice media. Their results also show that higher production of gliotoxin was observed on a rice medium, with a gliotoxin concentration of 5.33 ppm.

3.3 Effect of incubation periods

The effect of different incubation periods (between 3 and 14 days) on gliotoxin production using rice medium was investigated. The results indicated that the *A. fumigatus* isolate AF-5 has the ability to produce gliotoxin in all incubation periods (Fig. 5), but to determine which period is the best for gliotoxin production the toxin extracts were analysed by HPLC. HPLC analysis revealed that the maximum concentration of gliotoxin (115.6 ppm) was obtained after 10 days, but that this decreased to 78.8 ppm after 12 days (Fig. 6).

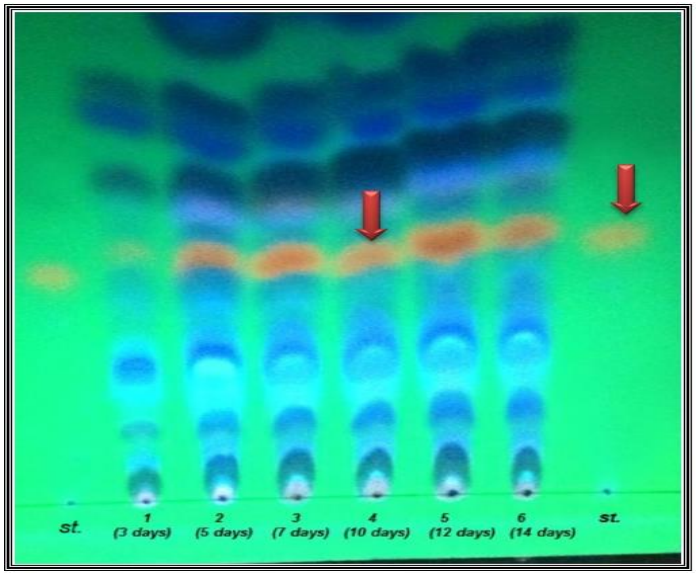

Figure 5: Detection of the ability of *A. fumigatus*AF-5 isolate to produce gliotoxin on a rice medium at different incubation periods.

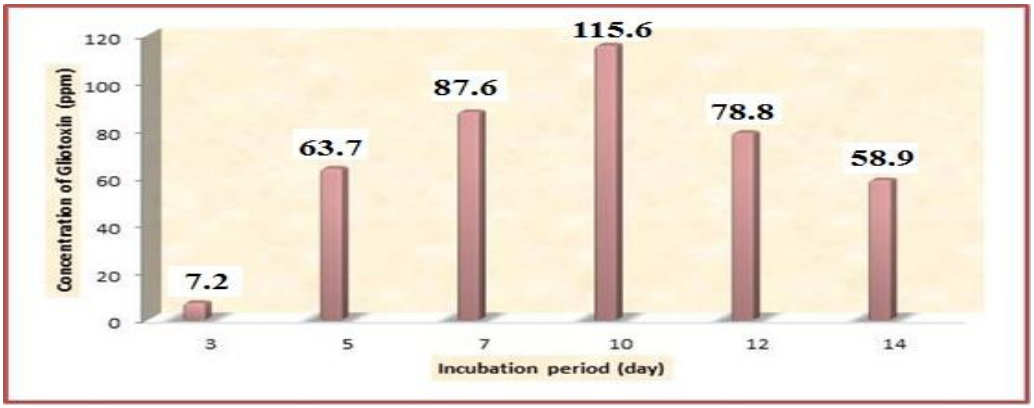

Figure 6: The effect of incubation period on the amount of gliotoxin production by *A. fumigatus* isolate AF-5 on a rice medium, with a moisture ratio of 5:1 (w:v), inoculum size of 1 ml, when incubated at 28 °C.

Other studies have shown that gliotoxin production decreases with time, with most growth occurring in the exponential phase. The subsequent decline in productivity may be due to catabolism, and instability of the compound under the culture conditions used, or, perhaps, to the production of an antagonist late in culture [16].

Richard and his colleagues [8] showed that maximum gliotoxin production occurs after 8 days of incubation. Kosalec and his colleagues [17] also showed that the concentration of gliotoxin increased progressively during incubation, with the average being 4.06 ppm after 3 days but 8.59 ppm after 12 days at 37 °C.

3.4 Effect of moisturising ratio

The moisture content of the culture medium affects the physiology of the microorganisms and we therefore attempted to find out the optimum moisture level of the medium for gliotoxin production. To check the influence of moisture content, the rice medium was moistened with different ratios of distilled water ranging between 5: 0.5 – 5: 2.5 (w/v).

Chemical analysis by TLC indicated that most gliotoxin was produced by the AF-5 isolate at a 5:1 to 5: 2.5 moisturising ratio (Fig. 7). Further analysis by HPLC revealed that the highest concentration of gliotoxin (110.1 ppm) was achieved with a moisturising ratio of 5: 1 w/v. At a ratio of 5: 0.5 w/v, however, the AF-5 isolate did not grown at all and thus there was no gliotoxin production (Fig. 8).

The mechanism by which the moisture level influences gliotoxin production in SSF relates to the fact that the solid substrates used in SSF are insoluble in water. This means that moisture has to be applied to the substrate particles in order for this to be used by microorganisms for their growth and their metabolic activities (including, in this case, production of gliotoxin). Indeed, microbial cells require about 70-80% moisture content for new cell biosynthesis and the moisture level is known to be a significant limiting factor affecting stability, biosynthesis and secretion of fungal metabolites [18].Thus, the degree to which the substrate is hydrated has an important influence on the growth of the fungi and subsequently the toxin production [19].

The optimum moisture level is closely related to other parameters, such as nature of the substrate, the organism and the studied metabolite. If the moisture level is less than the optimum the solubility and swelling capacity of the substrate may be diminished, which results in high water tension and decreased growth and metabolite production.

At moisture levels higher than the optimum, meanwhile, the associated reduction in the space between particles (steric hindrance) reduces porosity, results in a gummy texture and impairs oxygen transfer, together serving to reduce metabolite biosynthesis [20].

Dheeb (2013) recorded that the best moisture ratio for the production of gliotoxin by *A. fumigatus* under SSF in a rice medium was 20%. Richard and his colleagues (1989), meanwhile, found that the highest level of gliotoxin production (5.33 ppm) was when the moisture content was 50%.

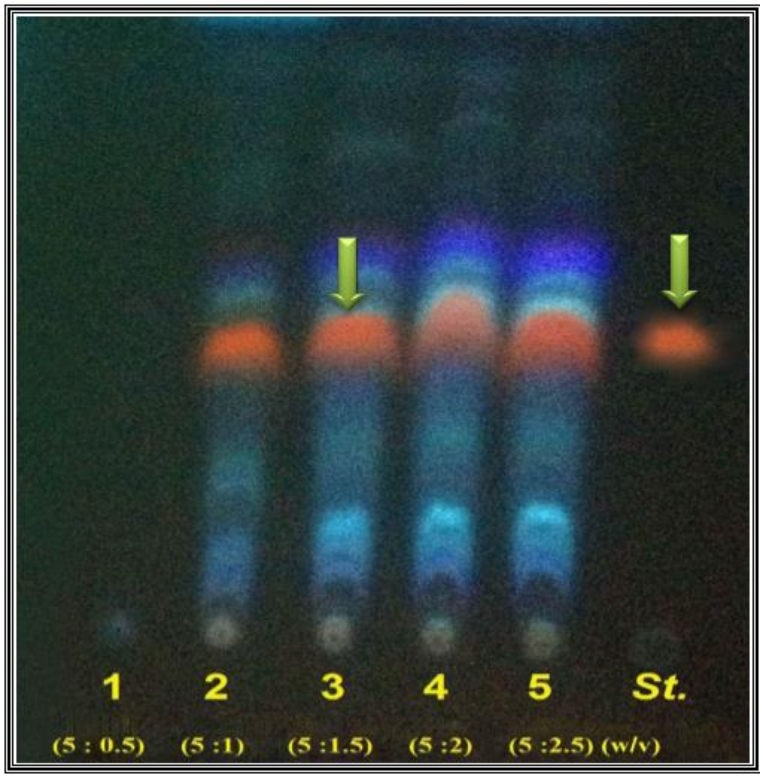

Figure 7: Detection of the ability of *A. fumigatus* isolate AF-5 to produce gliotoxin at different moisture ratios.

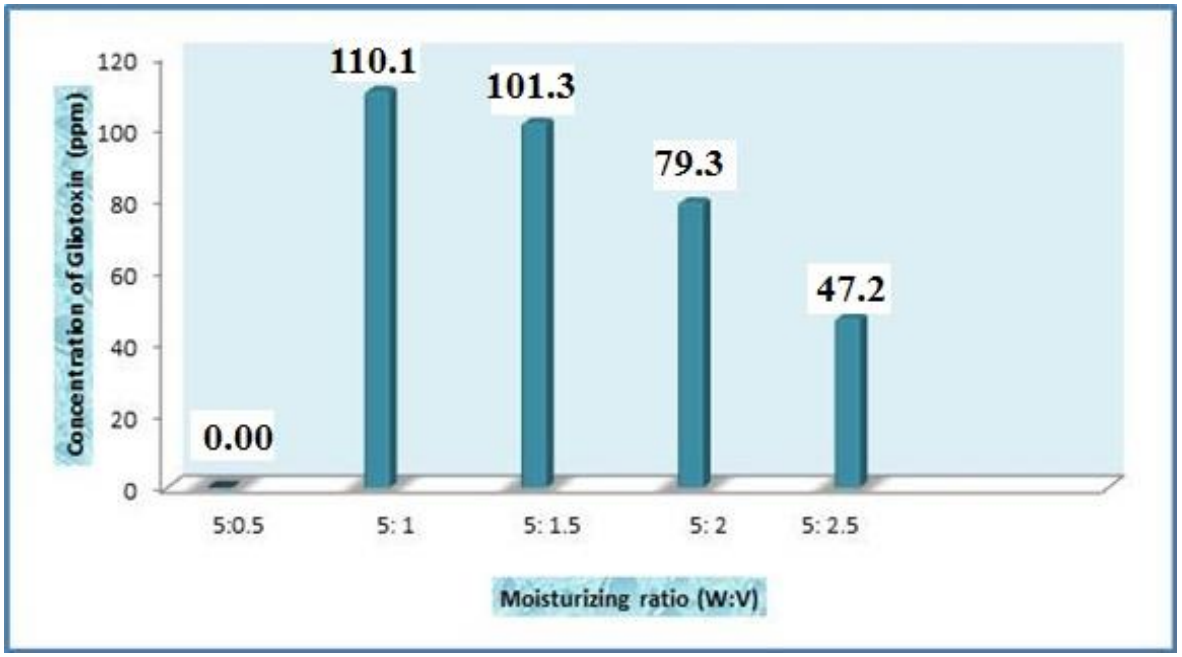

Figure 8: Effect of different moisture ratios on gliotoxin production from *A. fumigatus* isolate AF-5 in a rice medium, with an inoculum size of 1 ml, and when incubated at 28 °C for 10 days.

3.5 Effect of temperature:

Temperature is another factors affecting gliotoxin production. In this study, although gliotoxin production occurred at various temperatures (28, 32, 37 and 42 ºC), the optimum temperature was found to be 37 °C. This resulted in a concentration of 121.8 ppm (Figs. 9 and 10). Temperature is known directly to affect all vital events in the cell by influencing the genetic material and enzymes and lipids in the cell membrane, which in turn influences the extent and speed of growth [21].Belkacemi and his colleagues [22] also concluded that a temperature of 37 °C appeared to be optimal for gliotoxin production *in* v*itro* and that increasing or decreasing the amount of carbohydrates or reducing the temperature below 37 °C caused a decrease in gliotoxin production and biomass. Similarly, Kosalec and his colleagues [23] when comparing gliotoxin production at 25 °C to that at 37 °C, showed that the concentration doubled at the higher temperature. Richard and his colleagues [8] meanwhile, reported that higher production of gliotoxin was observed from a rice medium at 28 °C, with a concentration of gliotoxin of 5.33 ppm. Similar results were obtained by [24].

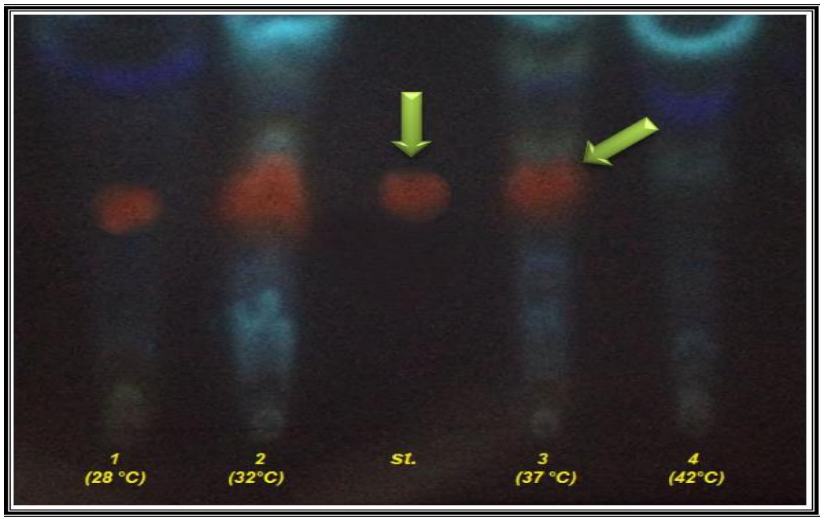

Figure 9: Detection of the ability of *A. fumigatus* isolate AF-5 to produce gliotoxin at different temperatures.

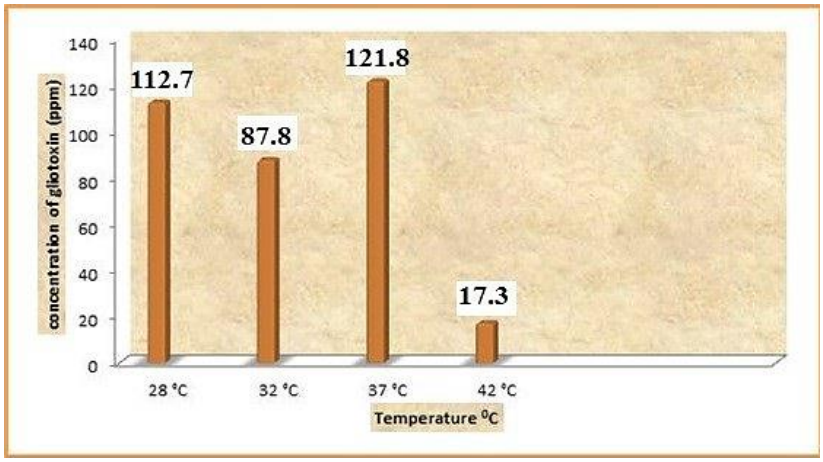

Figure 10: Effect of temperature on gliotoxin production from *A. fumigatus* isolate AF-5 in a rice medium, with a moisture ratio of 5:1 w/v, an inoculum size of 1 ml, and when incubated for 10 days.

3.6 Effect of inoculum size on gliotoxin production

The effect of different inoculum sizes (1×10^6– 8×10^6) on gliotoxin production was studied. The results of fungal growth on the rice medium indicated that good fungal growth was obtained for all the different inoculum sizes used (Fig. 11), but the most extensive fungal growth was obtained with an inoculum size of 1.5 ml. Different intensities of brown colour (representing gliotoxin) were observed when TLC was used to detect the presence of gliotoxin in the culture extract (Fig. 12).

HPLC was used to determine the gliotoxin concentration in the culture extract. The results (Fig. 13) revealed that the highest level of gliotoxin was observed when 1.5 ml (6×10^6) of inoculum size was used. This led to a gliotoxin concentration of 134.7 ppm. If the inoculum size is more than this level, the fungi initially grow very strongly but this is thought to result in a nutritional imbalance in the medium, or to the nutrients being used up before the fungi are physiologically ready to start toxin production. Inoculum sizes below the optimum level, meanwhile, are likely to result in lower production of gliotoxin due to insufficient fungal biomass.

At optimum inoculum sizes the right proportions of nutrient and oxygen will be available for growth. Therefore, there is a need to use an appropriate number of spores so that the fungus can grow and cover most of the particles of the solid substrate without the emergence of competition for nutrients [25].

Richardand his colleagues [8] found that the highest level of gliotoxin production (5.33 ppm) was obtained by *A. fumigatus* under SSF on rice medium when the inoculum size was 1 ml.

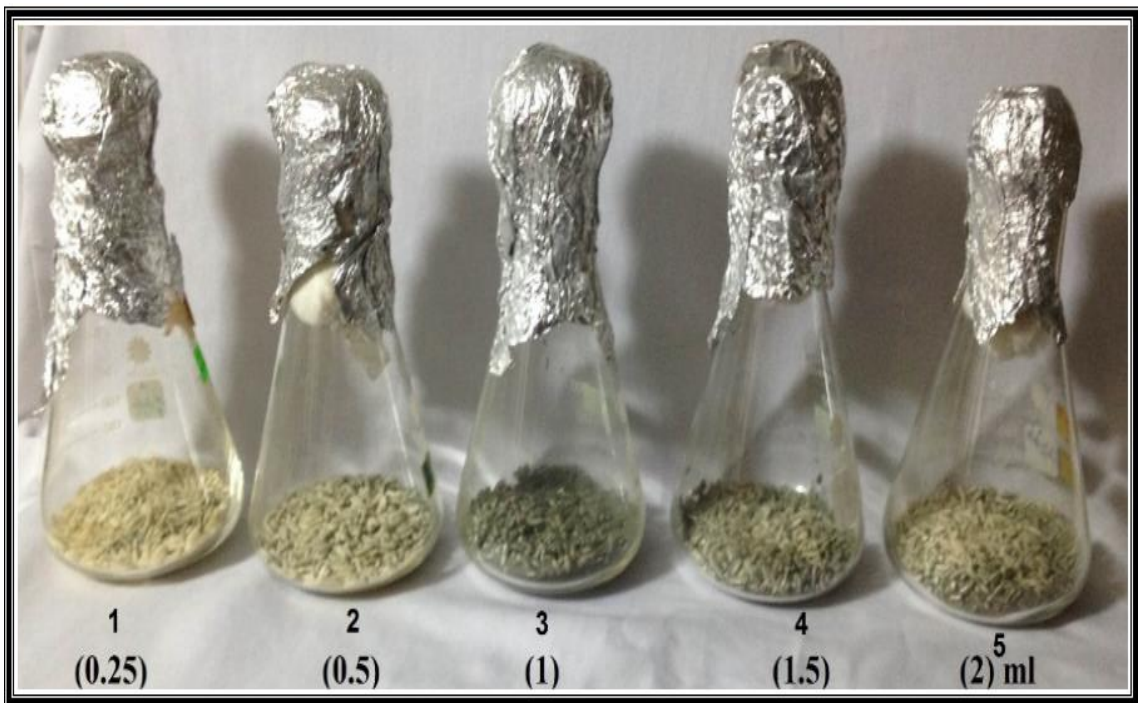

Figure 11:*A. fumigatus* isolate AF-5 grown on a rice medium with different inoculum sizes, a moisture ratio of 5:1 (w/v), when incubated at 37 °C for 10 days.

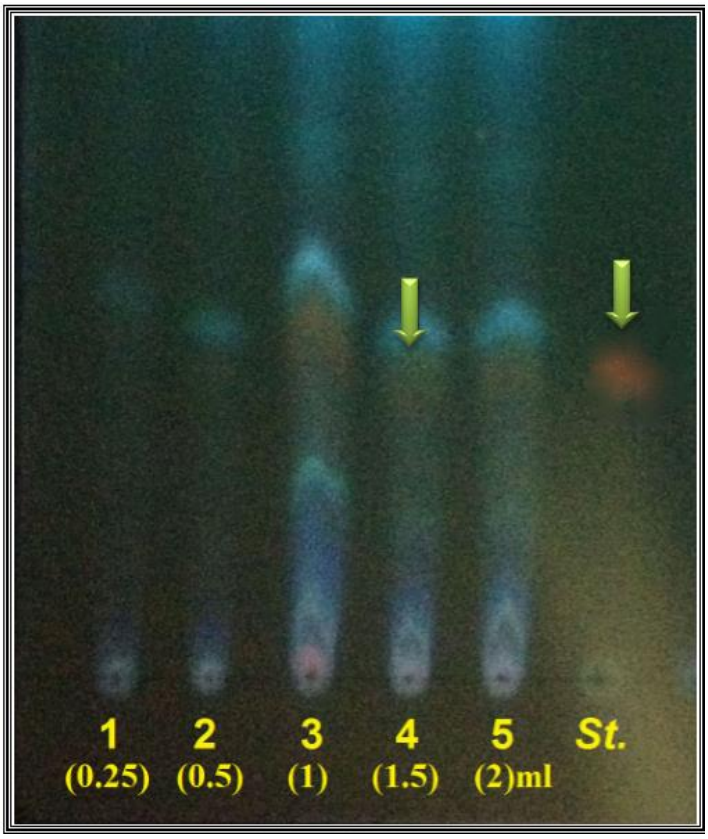

Figure 12: Detection of the ability of *A. fumigatus* isolate AF-5 to produce gliotoxin at different inoculum sizes.

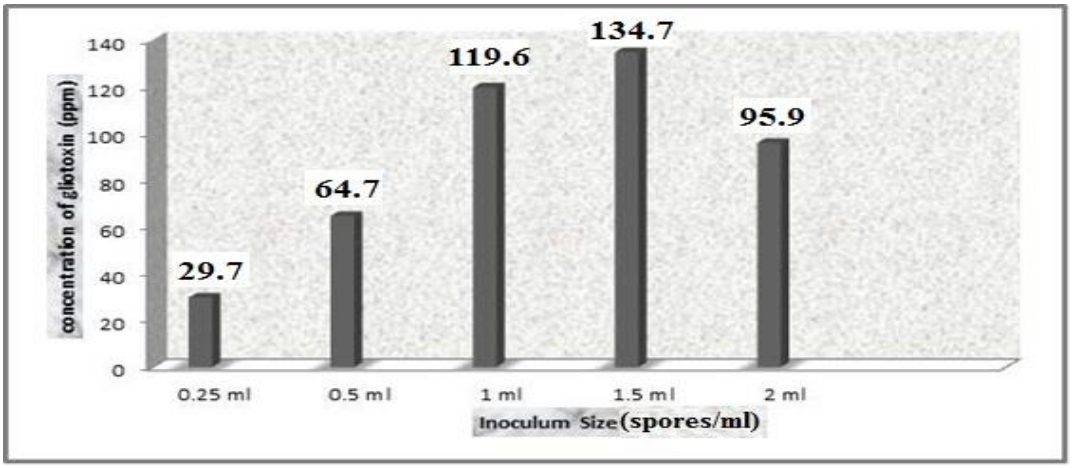

Figure 13: Effect of inoculum size on gliotoxin production from *A. fumigatus* on a rice medium, with a moisture ratio of 5:1 (w/v), and when incubated at 37 °C for 10 days.

3.7 Purification of gliotoxin by filtration and by elution of the Sep-Pak column

Filtration and Elution

Gliotoxin was purified from crude extract of *A. fumigatus* isolate AF-5after production using SSF under the optimum conditions set out above.

Purification of gliotoxin was achieved by two steps including: filtration and solid phase extraction. The crude extract was filtered using a 0.45 µmmillipore filter to remove debris. Then, to obtain pure toxin, the Sep-Pak columns were eluted using different organic solvents (hexane, ethyl acetate, chloroform and methanol). The results of the elution process indicated that clear extractfractions were obtained in each elute in comparison to the crude extract.

3.8 Assessment of the purity of Gliotoxin by TLC and HPLC

To detect gliotoxin and its purity and concentration in each of the organic extracts, TLC and HPLC was used. the results of the TLC analysis revealed that the highest purity of gliotoxin was evident when the Sep-Pak column was eluted with ethyl acetate, with no spots being observed when the other elutes were tested. Many spots were observed in both the crude and the filtrated extract, however, indicating that purification by eluting the Sep-Pak column was successful (Fig. 14).

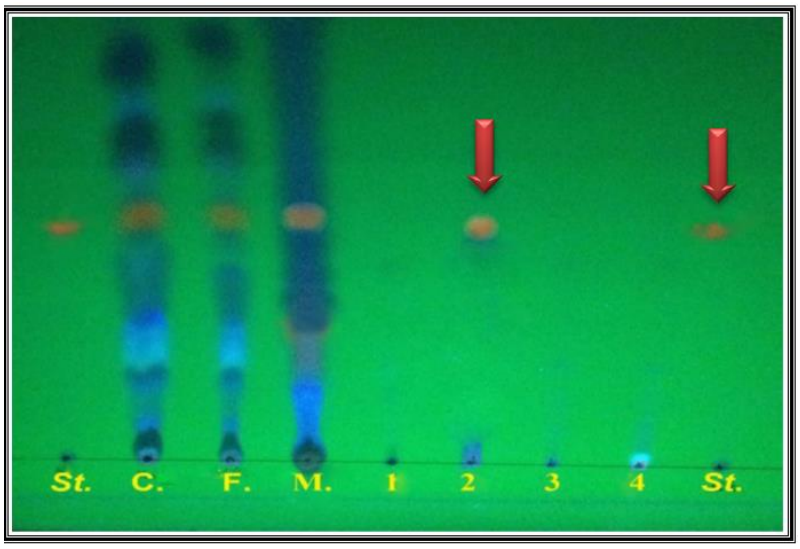

Figure 14: Detection of the gliotoxin by TLC plate, St: Standard gliotoxin**, C:** Crude extract**, F:** Filtered extract by millipore filters 0.45 µm**, M:**Methylene chloride**, 1:** Hexane**, 2:** Ethyl acetate**, 3:** Chloroform**, 4:** Methanol.

HPLC analysis was used to confirm the purity of gliotoxin and to determine its concentration compared to standard gliotoxin. The analysis revealed that the solvent system composed of methanol /water at ratio (43:57 v/v) was the most appropriate, because it gave one peak at a retention time of 10.2 min., which compares well with standard toxin (Fig. 15). These results differ from those of Richardand his colleagues [8] who used HPLC analysis with a column length of 10 cm x 4.6 mm to give a retention time of 4.8 min. This difference may be due to the difference in the length of the column that was used in the separation.

Belkacemiand his colleagues [25,26,27,28] used HPLC to detected gliotoxin, using a mobile phase of 50% methanol 50% deionized-distilled water. The amount of gliotoxin in the samples was calculated from the standard curve.

Zhang [29] indicate that using the HPLC method for the detection of gliotoxin content has the advantages of being fast, accurate and reproducible, and can lead to an average recovery of as much as 99.22%, when using optimal conditions (column, column temperature, mobile phase, wavelength and correlation coefficient).

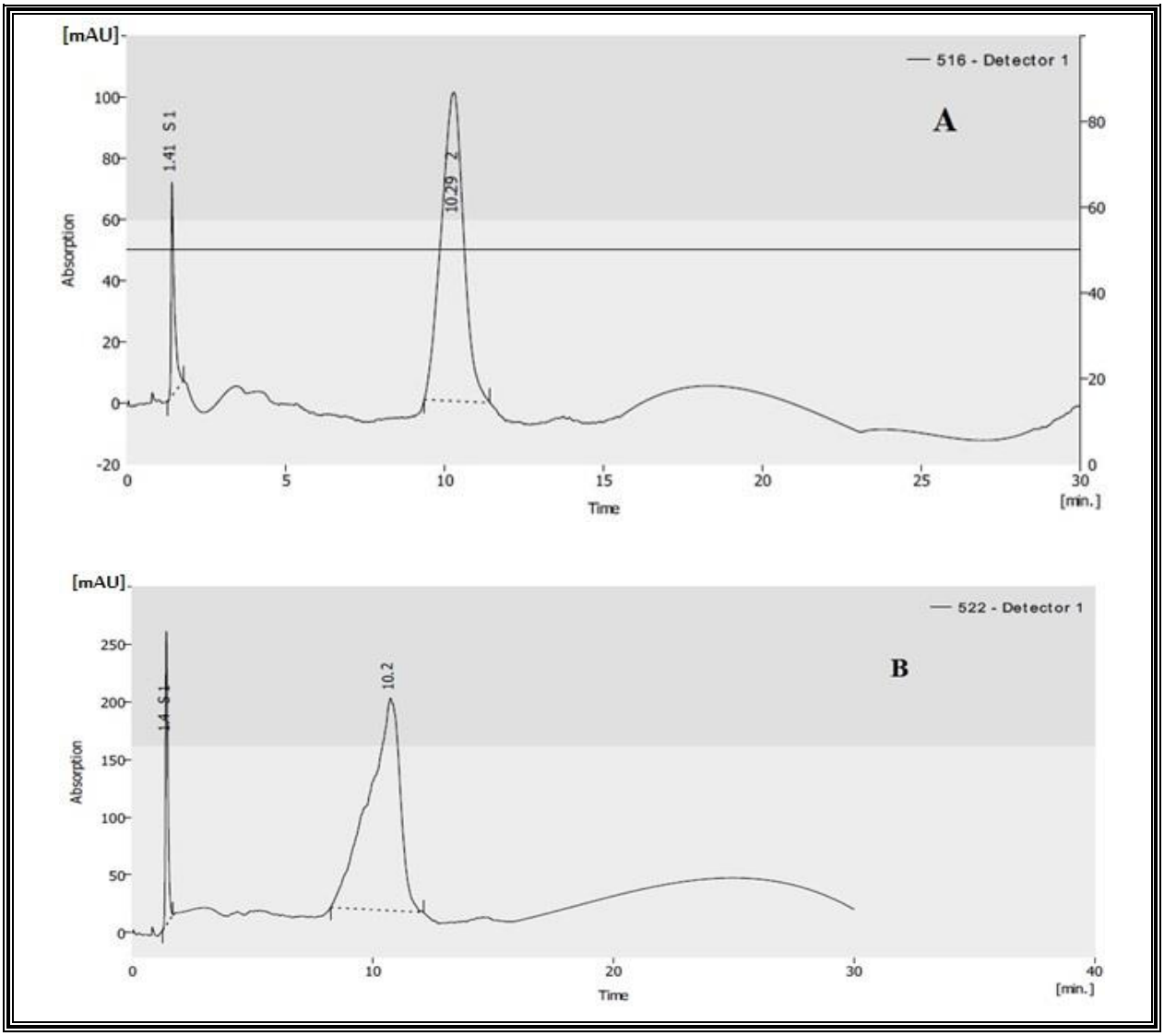

Figure 15: HPLC analysis(A) Detection of standard gliotoxin (1000 ppm).**(B)** Detection of gliotoxin in ethyl acetate extract.

4. Conclusions

1. Not all *A. fumigatus* isolates have the ability to produce gliotoxin.
2. The AF-5 isolate was the highest gliotoxin producer on a rice medium, which showed higher production than barley, corn and wheat media.
3. The optimum conditions for gliotoxin production were:- moisturising ratio of 5:1 (w: v) with distilled water, inoculum size of 6×10^6 spores and incubation at 37 ºC for 10 days.
4. Purification processes, included filtration and solid phase extraction, were used to remove debris from the crude extract and give pure gliotoxin.

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References

[1] Abdullah, A. L., Tengerdy, R. P. and Murphy, V. G. (1985). Optimization of solid state fermentation of wheat straw. Biotechnol. Bioeng. (27):20 - 27.

[2] Abu-Seidah, A.A., 2003. Secondary metabolites as co-markers in the taxonomy of Aspergilli. Acta Microbiol. Pol. 52 (1), 15 –23.

[3] Anitha, R. and Murugesan, K. (2005). Production of gliotoxin on natural substrates by *Trichoderma virens*. J. Basic Microbiol. 45 (1), 12–19.

[4] Belitz, H-D., Grosch, W. and Schieberle, P. (2009). Cereals and cereal products. In: Belitz H-D., Grosch W, Schieberle P (eds) Food chemistry, 4th edn. Springer, Berlin: 670–675.

[5] Belkacemi, L., Barton, R. C. , Hopwood V. and Evans E. G. V. (1999). Determination of optimum growth conditions for gliotoxin production by *Aspergillus fumigatus* and development of a novel method for gliotoxin detection Medical Mycology 1999, 37, 227–233.

[6] Choudhary, G. P. (2011). Biodeterioration in Emblica based Medicinal products and their Aflatoxin contamination. Anc Sci Life. 30(3):65-71.

[7] Denning, D. W., Pleuvry, A. and Cole, D. C. (2013). "Global burden of chronic pulmonary aspergillosis complicating sarcoidosis". European Respiratory Journal. 41(3): 621–6.

[8] Richard, J.L., Lyon, R.E., Ross, P.F., 1989. Use of thin layer chromatography for detection and high performance liquid chromatography for quantitating gliotoxin from rice cultures of Aspergillus fumigatus fresenius. Mycopathologia. 107 (2), 145–151.

[9] Dos-Santos, V. M., Dorner, J. W. and Carreira, F. (2003). Isolation and toxigenicity of *Aspergillus fumigatus* from moldy silage. Mycopathologia. (156):133–138.

[10] Eichner,R. D.,AlSalami,M.,Wood,P.R.andMullbacher,A.(1986). Theeffectofgliotoxin upon macrophage function.IntJ Immunopharmacol. 8:789-797.

[11] Kosalec, I., Pepeljnjak, S., Jandrlić. M., 2005. Influence of media and temperature on gliotoxin production in Aspergillus fumig-atus strains. Arh. Hig. Rada. Toksikol. 56(3), 269–73.

[12] Furqan, H. (2005). The effect of substrate topography and mechanical strain on the regulation of neurite development in neuron cells. Ph.D. Thesis, University of Georgia,Athens, Georgia.

[13] Kosalec, I. and Pepeljnjak, S. (2005). Mycotoxigenicity of clinical and environmental *Aspergillus fumigatus* and *A. flavus* isolates. Acta Pharm. (55):365–375.

[14] Kupfahl, C., Heinekamp, T., Geginat, G., Ruppert, T., Härtl, A., Hof H. and Brakhage, A. A. (2006). Deletion of the gliP gene of *Aspergillus fumigatus* results in loss of gliotoxin production but has no effect on virulence of the fungus in a low-dose mouse infection model. Mol Microbiol.62(1):292–302.

[15] Kwon-Chung, K., Janyce Sugui A.J., 2009. What do we know about the role of gliotoxin in the pathobiology of *Aspergillus fumigatus*? Med. Mycol. 47(Suppl 1): S97–103.

[16] Lewis, R. E., Wiederhold, N. P., Lionakis, M. S., Prince, R. A. and Kontoyiannis, D. P. (2005). Frequency and species distribution of gliotoxin-producing *Aspergillus* isolates recovered from patients at a tertiary-care cancer center. J Clin Microbiol. 43:6120–6122.

[17] Norton, C. F. (1986). How to grow and study microorganisms. In: Microbiology. 2nd edition, Addison - Wesley Publishing, USA, PP. 165 - 193.

[18] Oliver, G., Holgado, A. P. and Salim, R. (1982). Dimorphism in *Candida albicans* effect of cycloheximide and acridine orange on germ tube formation. Mycopath. 79: 43-47.

[19] Orciuolo,E.,Stanzani,M.,Canestraro,M.,Galimberti,S.,Carulli,G.,Lewis,R.,Petrini,M. and Komanduri, K. V.(2007). Effects of *Aspergillus fumigatus* gliotoxin and methylprednisolone on human neutrophils: implications for the pathogenesis ofinvasive aspergillosis.JLeukocBiol. 82:839-848.

[20] Pandey, A. (1992). Recent developments in solid state fermentation. Proc. Biochem., 27 (2): 109- 117.

[21] Pandey, A., Soccol, C. R., Selvakumar, V. T., Soccol, N. and Krieger, J D. (1999). Recent developments in microbial inulinases, Its production, properties and microbiol applications. Appl. Biochem. Biotechnol. (81): 35 - 52.

[22] Rathakrishnan, P. and Nagarajan, P. (2011). Red gram husk: A potent substrate for production of protease by *Bacillus cereus* in solid - state fermentation. Int. J. Chem. Tech. Res. 3 (3): 1526 - 1533.

[23] Richard, J. L. and Mary, C. D. (1995). Production of gliotoxin during the pathogenic state in turkey poults by *Aspergillus fumigatus* fresenius .Mycopathologia .129:111-115.

[24] Dheeb, B. I. (2013). Study on the pathogenicity, cytotoxicity and virulence factors of some clinical of *Aspergillus fumigatus* isolates and the use of RAPD markers to distinguish them. Ph.D. Thesis. College of Science, Tikrit University. Iraq.

[25] Vigushi, D. M., Mirsaidi, N., Brooke, G., Sun, C., Pace, P., Inman, L.,Moody, C. J. and Coombes, R. C. (2004). Gliotoxin is a dual inhibitor offarnesyl transferase and geranylgeranyltransferase I with antitumor activityagainst breast cancer in vivo. Med Oncol. 21(1):21-30.

[26] Waring, P., Eichner, R. D., Müllbacher, A. and Sjaarda, A. (1988). Gliotoxin induces apoptosis in macrophages unrelated to its antiphagocytic properties. J Biol Chem 263:18493-18499.

[27] Weindling, R. and Emerson O. H. (1936). The isolation of a toxic substance from a culture filtrate of *Trichoderma*. Phytopathology 26:1068 –1070.

[28] Wild, C.P., Gong, Y.Y. 2010. Mycotoxins and human disease: a largely ignored global health issue. Carcinogenesis. 31 (1), 71–82.

[29] Zhang, X., Zeping, X. U., Yang, C., Wang, J. and Zheng W. (2012). Analytical Method of Gliotoxin Content by HPLC. Advanced Materials Research. vol. (581-582):46-49.