Improving Conditions for Gliotoxin Production by Local Isolates of *Aspergillus fumigatus*
تحسين الظروف من عزلات محلية من الفطر *Aspergillus fumigatus* لأنتاج مستويات عالية من الكلايوتوكسين

Anaam Fuad Hussain **Ghassan Mohammad Sulaiman*** **Batol Imran Dheeb****
Abdulkareem Jasim Hashim*** **Sara Haitham Seddiq******
College of Science/ Diyala University
* Department of Applied Sciences/ University of Technology
** College of education/ Iraqia University
*** College of Science/ University of Baghdad
**** College of Science for women/ University of Baghdad

انعام فؤاد حسين **غسان محمد سليمان*** **بتول عمران ذيب**** **عبدالكريم جاسم هاشم***** **سارة هيثم صديق******
كلية العلوم/ جامعة ديالى
*قسم العلوم التطبيقية / الجامعة التكنلوجية
**كلية العلوم/ الجامعة العراقية
***كلية العلوم/ جامعة بغداد
****كلية العلوم للبنات/ جامعة بغداد

E-mail: batoolomran@yahoo.com

Abstract

Thirty two isolates of *Aspergillus fumigatus* were obtained from a total of 44 samples of sputum, nose swab and tracheal aspirate from suspected patient with aspergillosis were also collected from February 2014 to June 2014. A morphological examination of *A. fumigatus* was first made with naked eye and at low magnification power of microscope after that detailed examination was done by measuring the dimensions of the microscopic structures, photographing the microscopic structures and using relevant literature. Results appeared conical-shaped terminal vesicles, uniseriate row of phialides on the upper two thirds of the vesicle, conidiophore stipes were short, phialides arrange uniseriate upper vesicle conidia and parallel to axis of conidiophore, produced in chains of spore basipetally from phialides, the chains of spore were borne directly in the absence of metulae and represented by septet and branching hyphae. The ability of *A. fumigatus* for GT production was investigated using thin layer Chromatography (TLC) and High Performance Liquid Chromatography (HPLC) techniques and results showed that GT was produced by 81.25% of *A. fumigatus* isolates. Optimum conditions for GT production by *A. fumigatus* 16 (AF-16) isolate were determined by submerged fermentation using Yeast extract sucrose medium. Results indicated that AF-16 isolate was the highest GT producer on Yeast Exract Sucrose medium with inoculum size 2×10^7 conidium and incubation at 32ºC for 15 days and the concentration of gliotoxin was (4511µg mL^{-1}).

Key words: *Aspergillus fumigatus*, Mycotoxins, Gliotoxin, TLC, HPLC, Czapek-Dox broth medium, yeast-extracted broth medium, RPMI medium.

الملخص

جمعت عينات من المرضى المتوقع أصابتهم بداء الرشاشيات إذ حصل على 32 عزلة من مجموع 44 عينة من القشع ومسحات الانف والقصبة الهوائية للمرضى المتوقع اصابتهم بهذا الداء جمعت العينات للفترة من كانون الثاني للعام 2014 لغاية حزيران 2014.عملت فحوصات مظهرية بالعين المجردة وعلى القوة الصغرى للمجهر لفطر *Aspergillus fumigatus* ثم تلاها الفحص الدقيق للفطر من خلال قياس أبعاد التراكيب المجهرية وتصويرها بالاعتماد على مصادر سابقة. أظهرت النتائج بوجود تراكيب مخروطية الشكل ذات نهابة حوصلية ذات صف واحد من الفياليدات (تسمى احيانا بالذنيبات) مرتبة على الثلث الثاني من الحوصلة، يتميز الحامل الكونيدي بكونه قصير وتترتب الكونيدات على الفياليدات في أعلى الحوصلة وتكون موازية للحامل الكونيدي، وتتولد الأخيرة (الكونيدات) بشكل سلسلة من الفياليدات إذ تتولد سلسلة السبورات هذه مباشرة بغياب الذنيبات الأبتدائية اذ تتكون بحاجز وهايفات متفرعة. كشف عن قابلية *Aspergillus fumigatus* على إنتاج الغلايوتوكسين بإستخدام تقنية كروموتوغرافيا الطبقة الرقيقة. أظهرت النتائج قابلية 25 عزلة(81.25%) من عزلات*A. fumigatus* على إنتاج السم بينما لم تظهر عزلات الكانديدا جميعها القابليةعلى الإنتاج. تمثلت الظروف المثلى لإنتاج الغلايوتوكسين لتخمرات الحالة السائلة بإستخدام وسط خلاصة الخميرة كمادة أساس للعزلة المنتجة الأكفأ التي يرمز لها AF-16وحجم اللقاح2 ×10^7سبوربدرجة حرارة 32 º م لمدة 15 يوما" إذ كان تركيزالغلايوتوكسين 4511ميكروغرام مل$^{-1}$باستخدام تقنية الكروموتوغرافيا السائلة ذات الإداء العالي.

الكلمات الدالة: المايتوتوكسينات، الفطر *Aspergillus fumigatus*، الغلايوتوكسين، TLC, تقنية الكروموتوغرافيا السائلة ، وسط خلاصة الخميرة، الوسط وسط RPMI Czapek-Dox broth

Introduction

Gliotoxin is produced by number of fungi including: *Aspergillus, Penicillium spp*. also by some species of *Gliocladium*, *Thermoascus* and *Candida* [1]. Gliotoxin (GT) primarily has immunosuppressive activity [2], and is now considered as a virulence factor in human and animal aspergillosis and has immunosuppressive properties and inhibits mammalian cell proliferation [3]. It is capable of inhibiting function and inducing apoptotic cell death in macrophages and thus may alter the immune response [4], GT has been detected in lung tissue samples of poultry [5], and from aspergillosis human sputum [6] where it may facilitate fungal persistence and colonization of tissue. In addition, GT has been implicated for the destruction of lung parenchyma in invasive aspergillosis [7]. Interest in Gliotoxin as a therapeutic agent was initiated when shown has anti-inflammatory properties *in vitro* [8]. Gliotoxin can be used as a drug in the treatment of non-alcoholic fatty liver disease by inhibition of the activity of the nuclear factor and activation of apoptosis through the inhibition of mitochondrial ATP synthesis, which leads to the hyperpolarization in the membranes of mitochondria [9]. GT had significant antitumor activity at 0.50 mg kg^{-1} as it is a novel, potent; inhibitors that target clearly defined points in the Hypoxia Inducible Factor HIF pathway. GT is limited by its toxicity; specificity in targeting HIF-1 makes it attractive molecules for the design of future chemotherapeutic agents [3]. It has a very good antibacterial, antifungal and ant parasitical properties [6,7,8]. Locally, the ability of *A. fumigatus* to produce GT has not been studied yet in sufficient manner, so this study was aimed to produce it at high level from local fungal isolate and to achieve the aim of this study, the below steps were followed: Screening the ability of *A. fumigatus* isolates to produce GT, as a virulence factor, determination of the optimum conditions for GT production by submerged fermentation and production. Also separation and partial purification of GT produced in optimal conditions and detection it by TLC and HPLC techniques.

Materials and Methods

Isolation and identification of *A. fumigatus* isolates

Forty four samples were collected from immunocompromised patients suspected have infection with Aspergillosis (by clinically identification of physicians) suffered from leukemia from chest and respiratory diseases specialized center (Ministry of health, Baghdad Governorate), Baqubah Teaching Hospital (Diyala Governorate), Alkadmiya Teaching Hospital (Baghdad Governorate)/ Iraq. The samples were 18 of sputum, 16 nose swabs and 10 from tracheal aspirate. Specimens were examined by optical microscope [10]. Sputum, nose swab and tracheal aspirate specimens were cultured on Sabouraud Dextrose Agar (SDA) supplemented with 0.04 mg mL^{-1} Chloramphenicol to inhibit the growth of bacteria, then incubated at 37°C and examined for 7 days [10], and after seven days of incubation, plate was observed for macroscopic characteristics such as colony diameter, exudates, colony reverse and the isolates were identified to the species level on the basis of microscopic characteristics including conidiophore, vesicle, metulae, phialides and conidia. For microscopic characteristics slides were stained with Lactophenol cotton blue [5] with using adhesive tape preparation in which a small piece of transparent-adhesive tape was touched to the surface of the suspected colony, then adhered to the surface of a microscopic slide [6,11].

Photographs were taken with Digital microscopical camera.

A morphological examination of species was first made with naked eye and at low magnification power of microscope after that detailed examination was done according to Ellis and his collages [5] by measuring the dimensions of the microscopic structures, photographing the microscopic structures and using relevant literature.

Pure cultures of isolates were maintained in SDA slant medium with glycerol 15% and these were tightly wrapped with parafilm and placed at 4°C in refrigerator as stock cultures, sub cultured was done every three months [9]**.** After the isolates were cultured on SDA at 37°C for 7 days, slants of fungal colonies were covered with 10 ml of sterile physiological saline solution for preparing spore suspension, and the suspensions were prepared by scraping the surface of the slants with the tip of a capillary tube and gently agitation of the surface. 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 hemocytometer by added one drop of the suspension to hemocytometer by Pasteur pipette, spores were calculated under high power 40X of light microscope using the following equation:

Concentration of spores = ($Z \times 4 \times 10^7$) /n spores mL^{-1}

Where n: total number of small squares Z: total number of spores [6].

Screening of *A .fumigatus* isolates for gliotoxin production

Inoculum Preparation

Gliotoxin production on Yeast Extract Sucrose (YES) medium was achieved according to Kosalec [12] with some modifications, Each of 32 *A. fumigatus* isolates (duplicate for each isolate) were grown on Sabouraud Dextrose Agar (SDA) plates for 2 days at 37°C and the conidia were then harvested with sterile saline with 0.1% polysorbate 80 (Tween®80) and adjusted to a concentration of 10^7 conidium mL^{-1} in distilled water based on haemocytometer counts. One milliliter volumes of this conidial suspension were used to inoculate 100 ml of liquid medium (YES) in 250 ml flasks. The cultures were incubated at 37°C for 7 days; flasks were shaken manually at least twice a day.

Extraction and detection of gliotoxin

After incubation time, the biomass was extracted with 50mL of chloroform and cut up in small pieces with electric homogenizer at 3.500 rpm for 10 min. The biomass was then filtered by Whatman No.1 filter paper in separate Buchner funnels and extracted with 50 ml of chloroform, and filtered through anhydrous Sodium Sulphate. Chloroform fraction was collected and evaporated to dryness at 60°C. Dried extracts were dissolved in 500µL chloroform and stored at 4°C until gliotoxin was analyzed [12].

Detection of GT was performed by Thin layer chromatography (TLC).Ten micro liters of each chloroform extract in methanol and of 500 μgmL^{-1} standard gliotoxin (prepared with concentration 1000 μgmL^{-1} by dissolving 5mg of the standard gliotoxin in 5ml of absolute methanol, and stored at -70ºC in deep freeze) in methanol, were spotted on Silica gel 60 plate with fluorescence indicator approximately 3cm from the bottom edge [13]. After development the TLC plate with a mobile-phase toluene: ethyl acetate: formic acid (5:4:1) as a solvent system. The plates were dried under warm air and used to screen the presence of fluorescent compounds by viewing the plates under the UV cabinet at wavelength 366 nm. Before that plate was heated for 10 minutes at 110 °C [14]. To detect the highest GT producer isolate, a technique based on account of Relative flow (R_f) was used where the spots marked and R_f value measured for standard gliotoxin and sample according to the following equation:

$$R_f = \frac{\text{Distance spot moved}}{\text{Distance solvent moved}}$$

The tested samples had been matched with standard. Sample spots color and shape were also compared with standard Spot [15].

Detection of gliotoxin by High Performance Liquid Chromatography (HPLC)

According to Kupfahl [13], this experiment was conducted at the Ministry of Sciences and Technology/ Treatment of Contamination Center. The conditions for the HPLC analysis were as follow:

Instrument: Sykam- Germany

- Analytical column: C18 (30 cm x4.6 mm)
- Loop: 50µl
- Wavelength: Det- UV- 254 nm
- The mobile phase: methanol: deionized distle water (70:30)
- Flow rate: 1ml/min.

Injection of 500 $\mu g\ mL^{-1}$of standard gliotoxin dissolved in the methanol as conducted to determine retention time RT and relative peak area. A standard curve of the relationship of peak area quantity (ppm) injected was constructed, the concentration of purified sample gliotoxin was calculated using standard curve of the pure compound.

Optimal Conditions for Gliotoxin Production

Effect of cultures media

Selected isolate of *A. fumigatus* was grown in each of three different media (Czapic Dox Broth CDB, YES and Ross well Park Memorial Institute RPMI 1640 medium). 100 ml of cultures of each media were inoculated with 1ml (1×10^7 conidium mL^{-1}) of spore suspension in 250ml flask (Duplicate cultures of each medium), and then incubated at 37°C for 12 days. After elapsing the incubation period the mycotoxin was extracted with chloroform, and stored in small vial at -70ºC until analyzed by HPLC.

Effect of temperatures

Eight flasks of YES medium were prepared as described above. Duplicate cultures of each flask were inoculated with 1ml (1×10^7 conidium mL^{-1}) of spore suspension, the all flasks were incubated at different temperature (28, 32, 37 and 42°C) for 12 days after elapsing the incubation period the toxin was extracted with chloroform and stored in small vial at -70 ºC until analyzed by HPLC.

Effect of inoculum size

Eight flasks of YES medium were prepared as described above with duplicate cultures of each flask were inoculated with different inoculum size 0.5, 1, 1.5 and 2 ml of spore suspension (1×10^7 conidium mL^{-1}). The all flasks were incubated at 32°C for 12 days after elapsing the incubation period the mycotoxin was extracted with chloroform, and stored in small vial at -70ºC until analyzed by HPLC.

Effect of incubation periods

Eight flasks of YES medium were prepared as described above with duplicate cultures of each flask were inoculated with 2 ml (2×10^7 conidium mL^{-1}) of spore suspension. All the flasks were incubated at 32°C. for different incubation times 6 ,9 ,12 and 15 days, after elapsing the incubation period the toxin was extracted with chloroform and stored in small vial at -70ºC until analyzed by HPLC.

Results and Discussion

Isolation and identification of *A .fumigatus* isolates:

A total of 44 samples of sputum, nose swab and tracheal aspirate from suspected patient with aspergillosis, were examined by direct 10% KOH. The cultural and microscopic examination illustrates that 42 isolates of the infections belonged to the fungus *Aspergillus* and *Aspergillus fumigatus* were in 32 samples, six isolates were *A. flavus* and two isolates were *A. niger* and *A. parasiticus* for each. Upon culturing on Sabouraud dextrose agar SDA, colonies of *A. fumigatus* appear fast grower; the colony size reach 7cm within a week when grown on SDA at 37°C, the colony seems powdery, the color at the first seems to be white then turning to dark greenish and changed to gray, reversed side of the colonies appeared pale yellow to tan Figure (1) which were in agreement with [14,15,16]. Microscopic examination as illustrated in figure (2) appeared conical-shaped terminal vesicles, uniseriate row of phialides on the upper two thirds of the vesicle. Conidiophore stipes were short, phialides arrange uniseriate upper vesicle conidia and parallel to axis of conidiophore, produced in chains of spore basipetally from phialides, The chains of spore were borne directly in the absence of metulae and represented by septet and branching hyphae [5,17,18].

Fig. (1): *Aspergillus fumigatus* grown on SDA at 37°C after 7 days of incubation

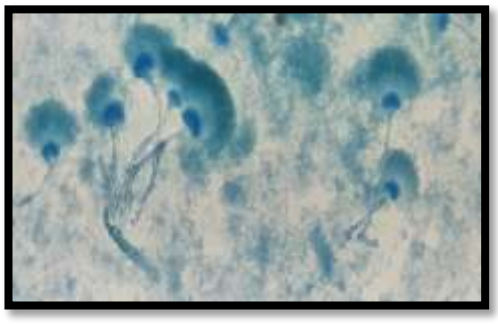


Fig. (2): Microscopic feature of *A. fumigatus* stained with Lactophenol cotton blue (40X)

Screening of *A. fumigatus* isolates for gliotoxin production

The ability of thirty two *A. fumigatus* isolates for GT production was determined using yeast extract medium as a submerged culture at 37°C after 7days of incubation. The chromatographically analysis using TLC indicated that, 26 isolates (81.25%) showed the ability to produce GT. When GT was visualized on TLC plates without spraying by reagent silver nitrate was appeared dark brown to red color under ultra violet light using UV cabinet at wavelength

366 nm by comparing the sample spots to the standard GT (same color, Relative flow (R*f*) and shape) figure (3). Results revealed that R*f* for both sample and standard GT was 0.75 and the isolate *A. fumigatus* NO.16 (A.F-16) was selected as the higher producer of GT.

TLC method was used with different mobile phase with same visualization of GT, In1970, Scott and his colleagues used (TLC) with suitable general solvent systems include toluene: ethyl acetate (90%): formic acid (6:3:1) and benzene: methanol: acetic acid (24:2:1) as mobile phase to detect GT produced from *Penicillium terlikowski* [24]. Belkacemi and his colleagues detected GT on TLC using methanol: chloroform (10:90 v/v) for revealing the GT spots [1]. Nouri, [18] used Methylene chloride: Methanol (97:3) to detect GT spots. In this study, TLC plate developed with a mobile-phase toluene: ethyl acetate: formic acid (5:4:1) as a solvent system [12,22]. The detection of GT with TLC was a simple, cost-effective technique often used as a mycotoxin screening assay with different solvents and when low detection limits were not required [15,23]. This study showed that YES broth with 4 % sucrose and 2 % yeast extract (YES) at the incubation temperature of 37°C and after 7 days were both suitable for rapid screening of *A. fumigatus* for GT production, because it takes no longer than 7days to detect GT in this cultures. YES was a very rich medium for GT production, this result was in agreement with Kosalec [12,24]. Although all the isolates used in this study were pathogenic but there was 25% of the isolates not producing GT in YES medium, not every strain was a GT producer because the efficiency of fungal isolates for mycotoxin production may affected with environmental and genetic factors [4,7,25].

The production of mycotoxins *in vitro* was mediated by substrates as source of its precursors and it was possible to increase mycotoxin production by using submerged cultures [4,12,26], therefore it was important to adjust production mechanisms of the secondary metabolite GT and optimization of nutrients to stimulate its production and using HPLC method to identify and quantify the purified GT as it was a very precise and highly automated quantification technique with high selectivity, sensitivity, good repeat ability and short analysis times [15,27].

Analysis of gliotoxin by High Performance Liquid Chromatography (HPLC)

Here we revealed that HPLC analysis for GT in YES culture extract of selected isolate with solvent system composed of methanol: water at ratio (70:30v/v) was very suitable, because it gave one peak with retention time 3.97 min in comparison with standard gliotoxin and there were no peaks evident at retention times immediately adjacent to that of GT figure (4), but in comparison with other studies, GT concentration in this study was about (3078.041µg mL^{-1}) at retention time 3.97min with column length (30 cm x 4.6 mm). Belkacemi also used HPLC to detect GT with mobile phase methanol: deionized-distilled water (50:50) and they revealed that (88.8%) of *A. fumigatus* environmental isolates tested by HPLC were found to be GT producers and the amount of GT in the samples was calculated from the standard curve [1,28]. Kupfahl and his colleagues showed that the HPLC effluent at the indicated retention time of GT 17.6 min. to strain of *A. fumigatus* which was grown in liquid RPMI medium for 7 days [13,29]. In several studies used the mobile phase methanol: water (43:57 v/v) for separation of gliotoxin from other secondary metabolites of rice medium was used [4,18,30], The results indicated that the concentration of the gliotoxin in the rice in these two separated studies was (1.2-5.3g gm^{-1}) and (122.6 ppm) and they represented the retention time for gliotoxin about 4.8 min and 10.2 min respectively.

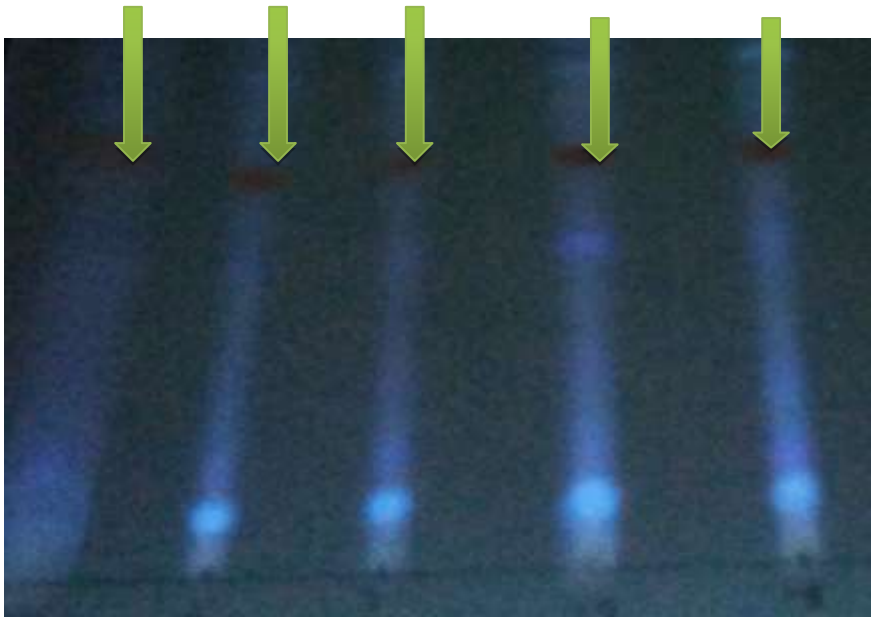
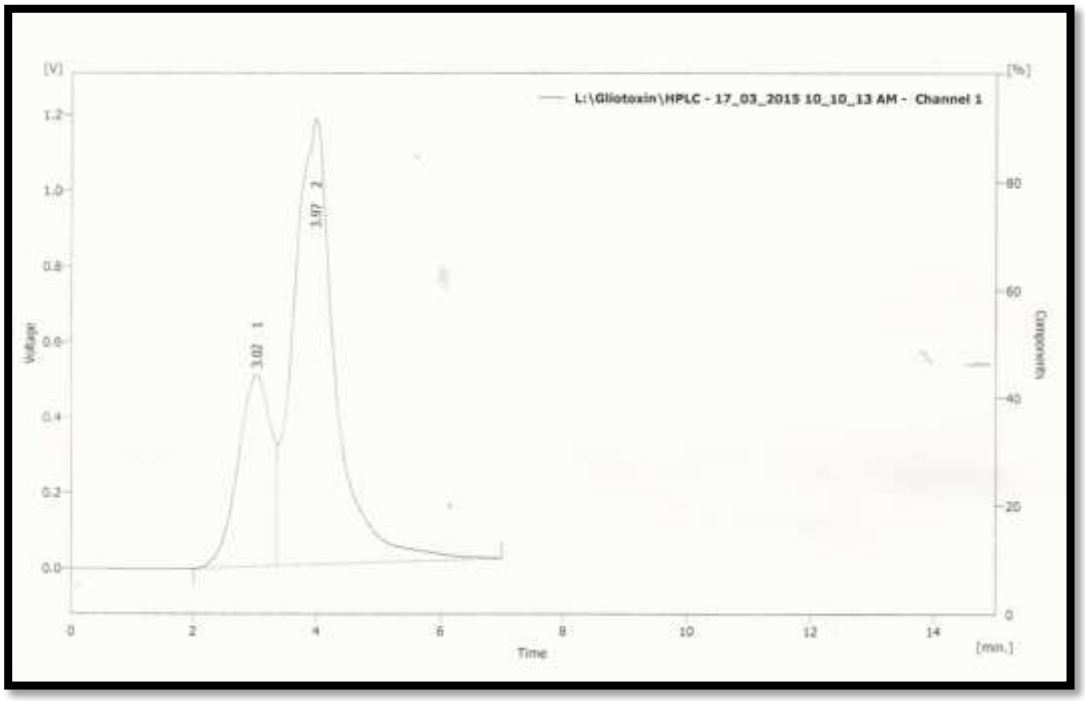
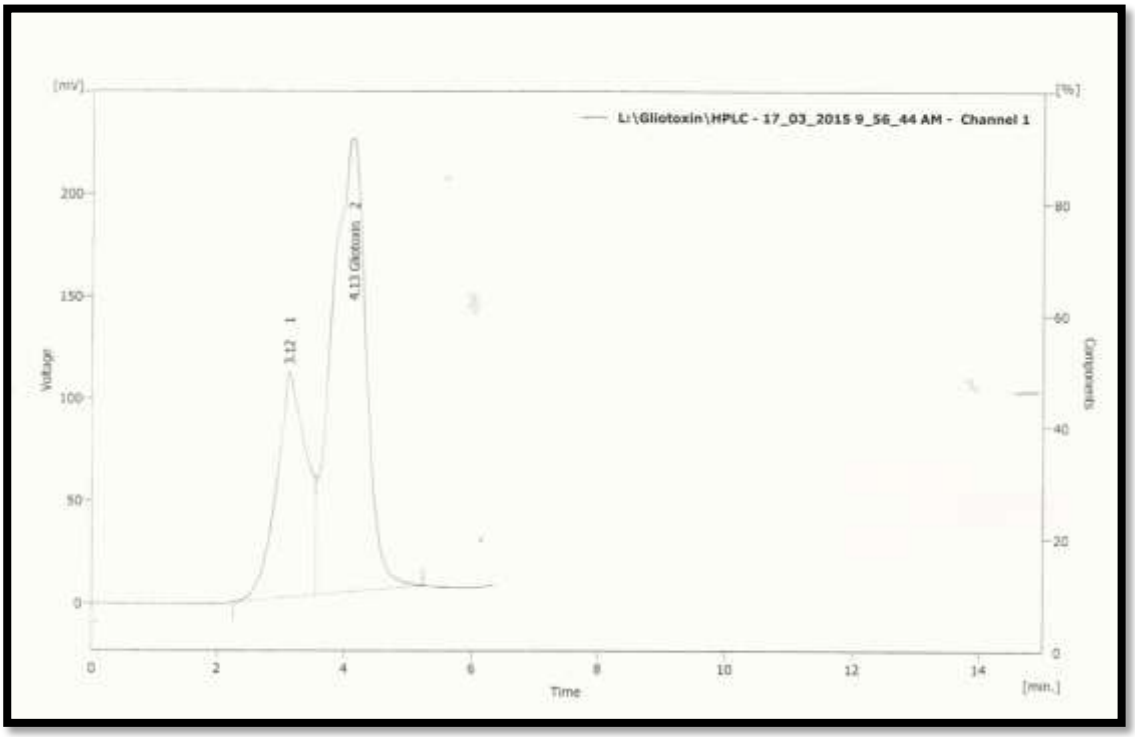


Fig. (3): Detection the ability of four *Aspergillus fumigatus* isolates for gliotoxin production on Yeast extract medium using TLC analysis method, the mobile phase was toluene: ethyl acetate: formic acid(5:4:1)



A



B

Figure (4): HPLC analysis (A) Detection of standard gliotoxin (500ppm), (B) Detection of extracted gliotoxin (sample), Peak 1:Retention time of the solvent (methanol), Peak 2:Retention time of gliotoxin

Production in optimum conditions

The effect of different cultures media on gliotoxin production

The results revealed that the selected *A. fumigatus* isolate grown on all the three media which used to produce GT but cultures media varied in their ability to induce GT production. The fungal growth was different depending on the type of culture media, the higher fungal growth (biomass) was observed in YES than in CDB and RPMI media. The fungal growth was almost identical in YES, CDB and RPMI media in spite of differences among these cultures components. HPLC analysis for methanolic extracts indicated that *A. fumigatus* can produce GT in all the three used media YES, CDB and RPMI with different concentrations (3384.65, 752.7and 138.4µg mL^{-1}) respectively as demonstrated in Figure (5). The results indicated that the composition of the medium used to grow *A. fumigatus* has an important influence on GT production and YES was the best culture medium for the growth and GT production Figure (5). and that may be due to that CDB contain less amount of carbohydrates (3% sucrose) as a source of Carbon as compared with YES (4% sucrose) [31]. In RPMI medium, concentration of GT revealed positive result by HPLC analysis with low concentration, suggesting that this growth medium lack some components which were vital for GT biosynthesis in spite of its containing of amino acids phenylalanine and serine, which were the very potent in stimulating GT production in molds [26,31] and the incubation at 37°C for 12 days might be suitable for secretion other extra metabolites which prevented GT production in high concentration.

Kupfahl and his colleagues showed that the HPLC effluent at the indicated retention time of GT 17.6 min to a strain of *A. fumigatus* which was grown in liquid RPMI medium for 7 days. They used GT extracted from *A. fumigatus* RPMI medium as a standard to detect it in *candida* spp supernatant extracts. By using submerged cultures for GT production, may be a suitable media for screening of other Epipolythiodioxopiperazines in large numbers of *A.*

fumigatus isolates as it was quick, easy, and with high purity [13,32,33].

The effect of temperatures on gliotoxin production

Temperature represents one of the factors that affect GT production, therefore; production was achieved at various temperatures (28, 32, 37 and 42ºC). The results showed that, normal fungal growth on YES medium was indicated when different temperature were used, but the higher fungal growth was observed at temperature 32ºC. HPLC was used to analysis extracted GT, and the optimum temperature for GT production by selected *A. fumigatus* isolate was found to be 32°C and GT concentration was 3763.33µgmL^{-1}, this concentration was the highest among the others which were 2379.075, 2242.42 and 1589.60µgmL^{-1} at 28, 37 and 42ºC, respectively Figure (6).

Temperature was affected in all vital events in the cell directly through influence in enzymes and lipids in the plasma membrane and in the genetic material then lead to influence in the quantity and speed of growth. Many studies suggested that a temperature of 37°C appeared to be optimal for GT production *in vitro* [1,12,34,35]. GT production was less at 42ºC than other temperature below and that depends on the interaction with other parameters at this experiment which were incubated time 12 days and YES medium, these two condition were suitable for GT production at 37°C [12,34,35], that means another material or mycotoxin may be released and inhibited the biosynthesis of GT.

The effect of inoculum size on gliotoxin production

A good fungal growth (biomass) on YES medium was obtained when different inoculum size (0.5ml of (1x10^7**),** 1 ml of 1x10^7**, 1.5** ml of 1x10^7 and 2 ml of 1x10^7**)** were used, but the higher fungal growth was obtained at 2 ml of inoculum size. To determine GT concentration in culture extract, HPLC for the analysis the extracted cultures was applied and the results were represented by Figure (7), the highest level of GT was observed when 2ml of inoculum size and GT concentration was 4233µg mL^{-1}. While, the rest extracts gave also good quantities of the mycotoxin represented by 318.07,2195 and 2063.55 µg mL^{-1} at 0.5, 1 and 1.5 ml of inoculum size, respectively. In broth media,1ml of 1× 10^7 conidia/ml was always used as an inoculum size to produce GT at high concentrations, these results were confirmed by an earlier study by Belkacemi and his colleagues in 1999, they revealed that 4·8 µg mL^{-1}of GT concentration in CDB medium at this volume and conditions [1,36]. Another study showed that GT concentration increased up to 8590 µgmL^{-1} with inoculum size 1ml at 37°C after 12 days incubation of this mold on YES medium [12]. However, high inoculum size led to the tremendous growth of the mold, resulting in nutritional imbalance in the medium or may be using up the nutrients before they were physiologically ready to start mycotoxin production. Low GT production below the inoculum size 2 ml may be due to insufficient fungal biomass. Inoculum size was one of biological method that effect on mycotoxin production; there was a relationship between inoculum size and mycotoxin production; in a suitable inoculum size, sufficient amount of nutrient and oxygen will be accessible for growth, therefore; there was a need to use an appropriate number of spores so that the fungus can grow and cover most of the particles of substrate without the emergency of a state of competition for nutrients available in a limited [18].

The effect of different incubation period on gliotoxin production

The effects of different incubation periods (6,9,12,15) days on GT production using YES medium were investigated. The results indicated that the selected *A. fumigatus* isolate has the ability to grow and produce GT in all incubation period. But to know which incubation period was the best for GT production the extracts were analyzed by HPLC, and the results of HPLC analysis revealed that the minimum concentration of GT (1223.97µgmL^{-1}) was obtained after 6 days. However, it increased up to 4511.08 µgmL^{-1}at the 15days. Results in Figure (8) showed clearly pronounced GT production with the increasing of incubation period up to 15 days. These results were in agreement with [12] who showed that the concentration of GT extracted from *A. fumigatus* in YES medium increased from 4060 µg/ml after 3 days of incubation time up to 8590 µg mL^{-1}after 12 days at 37°C. 4·8 µgmL^{-1} of GT concentration obtained from the same mold CDB medium, was achieved after 2 days incubation [13,36] other investigators showed that the concentration of GT obtained by the mold *A. fumigatus* on YES medium increased from 4060 µg ml^{-1} after 3days incubation up to 8590 µg mL^{-1}after 12 days at 37°C [12,37].

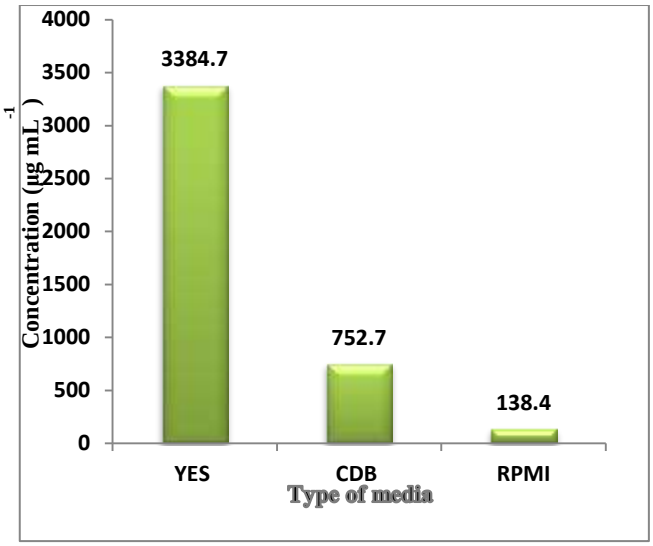


Fig. (5):Concentration of gliotoxin produced by selected *Aspergillus fumigatus* isolate on different cultures media, inoculum size 1 ml and incubated at 37°C for 12 days.

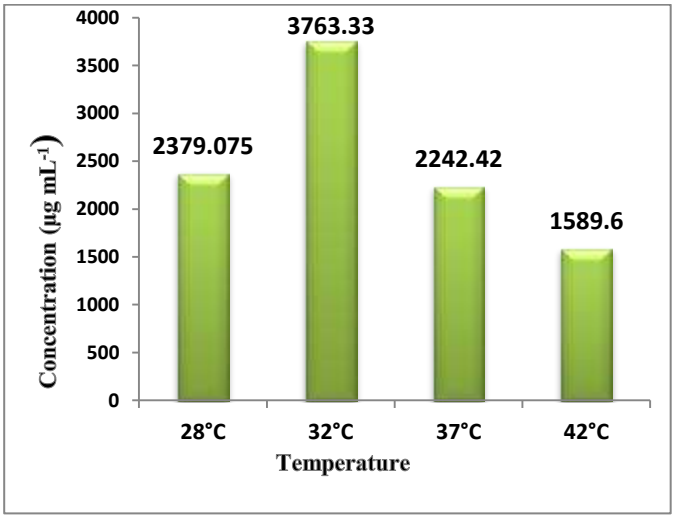


Fig. (6): Concentration of gliotoxin produced by selected *Aspergillus fumigatus* isolate on different temperature in YES medium, inoculum size 1 ml and incubated for 12 days.

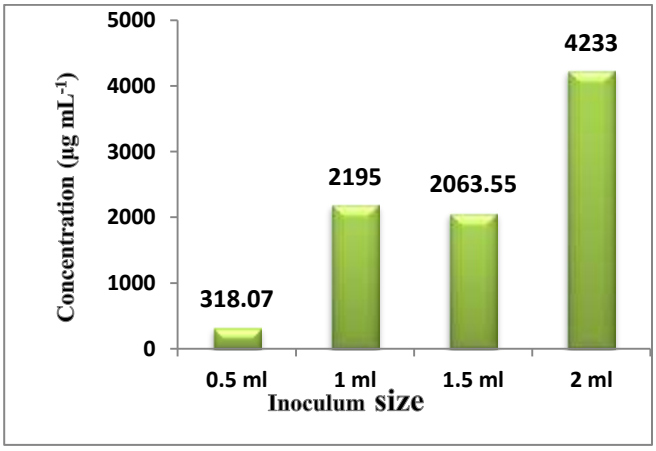


Fig. (7): Concentration of gliotoxin produced by selected *Aspergillus fumigatus* isolate in Different inoculum size on YES medium and incubated at 32°C for12 days.

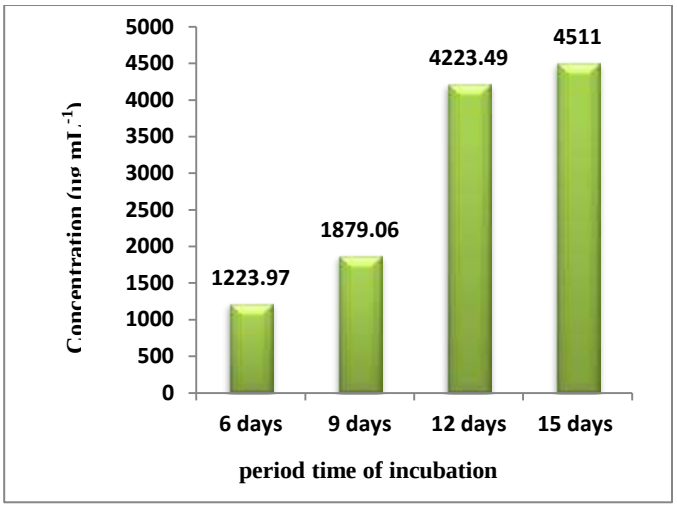


Fig. (8): Concentration of gliotoxin produced by selected *Aspergillus fumigatus* isolate in incubation on YES medium, different period time of inoculum size 2ml and incubated at 32°C.

Conclusions

Our study shows that production of gliotoxin *in vitro* is mediated by substrates as source of its precursors and broth media with 4 % sucrose and 2 % yeast extract (YES) is a very rich medium for gliotoxin production, and the selected isolate was the highest gliotoxin producer on YES medium with inoculum size 2×10^7 spores and incubation at 32ºC for 15 days and the concentration of gliotoxin was (4511µg mL^{-1}).

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