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Gliotoxin production from *Aspergillus fumigatus* isolated from Bovine Respiratory Infection

Noor Their Talib[1] , Dalia Abdalkareem Abdalshaheed[2]

ORCID: https://orcid.org/0000-0002-1645-48031 , https://orcid.org\0000-0003-1369-88262

[1]Microbiology Department college of Veterinary Medicine University of Diyala, Diyala, Iraq

[2]Microbiology Department college of Veterinary Medicine University of Baghdad, Baghdad, Iraq

Corresponding Email: dalia@covm.uobaghdad.edu.iq

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Abstract:

Background: The gliotoxin-producing capability of *A. fumigatus* isolates through High-Performance Liquid Chromatography (HPLC) determination. The results of this research revealed that among the 30 samples, *A. fumigatus* showed the highest yield of gliotoxin when cultivated on Yeast Extract Sucrose (YES) medium with a gliotoxin content of 23.823 µg /kg as determined by HPLC.

The primary goal of the study was to synthesize gliotoxin (GT) by *Aspergillus fumigatus*. The research resulted was conducted in the period between February 2022 and March 2023. Fifty nasal swabs were taken from animals in the Baghdad province with respiratory tract infection. All the samples received were grown on Czapek Dextrose Agar (CDA). The percentages of fungi isolated from nasal samples were as follows *A. fumigatus* (60%), *A. niger* (10%), *Aspergillus* spp. (16%), *Rhizopus* spp. (4%), Mucor spp. (2%), and Candida spp. (4%). To establish the identification of each isolate, both the macroscopic and the microscopic examinations were used.

Aims: The primary goal of the study was to synthesize gliotoxin (GT) by *Aspergillus fumigatus*.

R**esults**: Samples, which were collected from the nasal cavities of suspected animals having clinical symptoms from different areas in Baghdad Province, were cultured on czapek dox agar. The culture and microscope examination revealed 30 isolates for the infections were positive and identified as *Aspergillus fumigates*, whereas 20 isolates corresponded to other fungal species.

The findings of this investigation revealed that Yeast Extract Broth (YEB) supplemented with 4% sucrose and 2% yeast extract (YES) and incubated at 37 °C for seven days served as an efficient

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medium for the rapid assessment of *Aspergillus fumigatus* growth and gliotoxin (GT) biosynthesis, showing a yield of 23.823 µg/kg as determined by HPLC

Conclusions: The HPLC Technique was to achieve rapid, increased sensitivity, and specificity for gliotoxin detection.

Keyword: Gliotoxin, Czapek-Dox agar medium, HPLC, *Aspergillus fumigatus*, Yeast extract medium.

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Introduction:

Mycotoxins, which are synthesized by certain strains of fungi during the harvesting process, are highly toxic molecules with a rather low molecular weight (Mohammed *et al*., 2020). As persistent hazardous chemicals, that used by governments and international organizations of many of these pollutants, previously used to control a wide range of mould and yeast, led to an imbalance in the natural enemies in the environment, the low rate of which helps to maintain the pathogen (Fradi and Al-Araji, 2015). Gliotoxin is one of the other types of fungal metabolites recognized to be epipolythiodioxopiperazine (EPT) derivative with a disulfide bridge. Some fungal species, such as *Aspergillus fumigatus* (Reeves *et al*., 2004)), *Trichoderma virens* (Anitha *et al*., 2005)) and*Dichotomomyces cejpii* (Fan *et al*., 2016; Lin *et al*., 2019) produce this compound. (GT) discovered in *A. fumigatus* has been well studied and is prominent in its potent cytotoxic and immunomodulatory effects.

It has biological activities that are mediated by a variety of pathways, which include angiogenesis inhibition, anti-inflammatory response, immunosuppressive response, causing apoptosis, reactive oxygen species (ROS) production, and genotoxicity (Scharf *et al*., 2016).

Aspergillus fumigatus spores have the ability of attaching to pulmonary tissues of the epithelium, germinate, and generate a cluster of hyphae, which can lead to cavitation and potential destruction of the surrounding lung tissue (Tsunawaki *et al*., 2004). The cytotoxic properties of gliotoxin are mostly due to its capacity of the compound to inactivate major thiols on proteins (Hurne *et al*., 2000). In addition to this, GT also disrupts the assembly of NADPH oxidase, becoming the important host defense mechanism, thus it promotes fungal infection spread in the host (Tsunawaki *et al*., 2004; Nishida *et al*., 2005).

A. fumigatus controls the biosynthesis and metabolism of gliotoxin through a multi-gene cluster. This cluster contains the genetic instructions necessary for the enzymatic machinery responsible for regulating these critical processes.This cluster is coordinately expressed during gliotoxin biosynthesis (Gardiner *et al*., 2004; Gardiner and Howlett, 2005). GT and derivatives of GT

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are known to cause several animal mycoses (Kubodera *et al*., 2007; Patron *et al*., 2007) and can be involved in human fungal infections as well (Galagan *et al*., 2005).

Therefore, the aims of this research were to production of gliotoxin from *Aspergillus fumigatus* isolated from bovine respiratory infection.The continuous use of toxic chemicals by the government and industries in the environment which were aimed at controlling fungal populations has caused a lack of ecological balance. These pollutants have also lowered the concentration of the natural fungal antagonists, eventually increasing the survival and pathogenicity dominance in the environment of *A. fumigatus*.

Materials and Methods:

Collection of Samples

Fifty nasal discharges were collected from animals displaying respiratory infections in various locations within Baghdad province, during the period between from February 2022 to March 2023. The samples were directly placed on Cazpek Dextrose Agar and incubated at 37°C for 5 days. Furthermore, Petri dishes were incubated at 27±2°C for 7 days to observe the mycelia growth of pathogenic fungi. Identification of the fungal isolates was performed using lactophenol cotton blue staining, which is commonly employed for fungal microscopic examination (Minnat and Khalaf, 2019; Al-Ezzy and Abdulameer, 2021)), along with an assessment of the colony morphology on the culture plates.

Extraction and quantification of gliotoxin

The production of GT was according to the method described in the article by Kosalec *et al*. (2005); however, with some adjustments to optimize the production. All the thirty isolates of *A.fumigatus* were cultured in Czapek Dextrose Agar plates and allowed to incubate at 37°C for 5 days. The biomass of the fungus was isolated using 50 mL of chloroform after incubation. An electric homogenizer was utilized to homogenize the collected biomass at 3,500rpm in 10 minutes to get a homogenous suspension. The resulting mixture was filtered through Whatman No.1 filter paper, and then it was subjected to extraction with 2x25 mL of chloroform. The chloroform fractions were combined and passed through anhydrous Na2SO4 to remove any remaining water. A rotary evaporator on low pressure at 60 °C was used to concentrate the combined extract till it became dry.

The dry residue was re-solubilized in 500 uL chloroform and kept at 4 degC until the analysis of HPLC (High-Performance Liquid Chromatography). To perform the analytical step, the organic phase was dried up under a stream of nitrogen, and then it was reconstituted by 1000 µL of the mobile phase. Lastly, the quantitative determination of gliotoxin was done by injecting a 100 µL of the prepared sample in the HPLC system.

Determination of gliotoxin by HPLC

Determination of gliotoxin by HPLC in accordance to (Hussain, 2017), The research study was done at the Ministry of Science and Technology. All reagents and solvents were HPLC or

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analytical grade. Gliotoxin standards from Sigma-Aldrich (Germany) Company Figure (1).The conditions for the HPLC analysis were in Table (1)

Figure (1) Gliotoxin Standard

Table (1): Optimized Chromatographic Conditions for HPLC Analysis

Instrumental conditions	
Chromatographic Column	C18 column (30 cm × 4.6 mm)
Flow Rate	1.0 mL/min
Detection Wavelength	254 nm
Injection Volume	20 µL
Column Temperature	25 °C
Mobile Phase Composition	Methanol : Water (43:57, v/v) containing 1% trichloroacetic acid (TCA)

Injection of 500 µg/mL for each sample and standard of GT, following dissolution in methanol, was performed to ascertain persistence duration along with comparative peaks region. A standard curve depicting the correlation between peak area and the total amount of injected gliotoxin (ppm) was established, and the concentration of the pure GT sample was determined utilizing the typical curve of the pure substance as follows:

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$$\text{Concentration of sample ug/ml} = \frac{\text{Sample area}}{\text{Standard}} \times \text{con of standard} \times \text{dilution Factor}$$

Statistical Analysis

Means data values were displayed with the standard error (mean ± SE) and subjected to (ANOVA) followed by several means comparison.

Results:

Isolation of Fungi from Bovine Respiratory Samples

The current study allows for the distinguishing of molds and yeasts that cause respiratory infections in cattle. Samples, which collected from the nasal discharge of suspected animals having clinical symptoms from different areas in Baghdad governorate, were cultured on czapek dox agar. The culture and microscope examination revealed 30 isolates for the infections were positive and identified as *Aspergillus fumigates*; whereas 20 isolates corresponded to other fungal species as shown in (Table 2)

Table (2) Percentage of samples isolated from nose of cows

Fungal spp.	**Isolation No.**	**Percentage%**
Aspergillus fumigatus	**30**	**60**
Aspergillus spp.	**13**	**26**
Rhizopus spp.	**2**	**4**
Mucar spp.	**1**	**2**
Candida spp.	**4**	**8**
Total	**50**	**100%**

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The genus *Aspergillus* (*Aspergillus fumigatus*, *Aspergillus* spp *Aspergillus niger*) were counted for 60% , 16%, 10% , respectively .While *Rhizopus* spp.was 4% , *Mucar* spp. 2% and *Candida* spp. was 8% of the total samples.

Identification of *Aspergillus fumigates* Isolates

A.fumigatus can be identified according to its morphological features on the cultivated media (i.e., identified in both macroscopic and microscopic shapes).

Macroscopic Appearance

Macroscopic diagnosis of the fungus showed, *A.fumigatus*. Within a week, the fungus displayed a colony size of 7cm when cultivated on Cazpek Dextrose Agar at a temperature of 37°C.The appearance of the colony was powdery, starting with a white color, which later turned into a dark greenish shade before finally changing to gray. A pale yellow to tan color was noticed on the reverse side of the colonies figure (2).

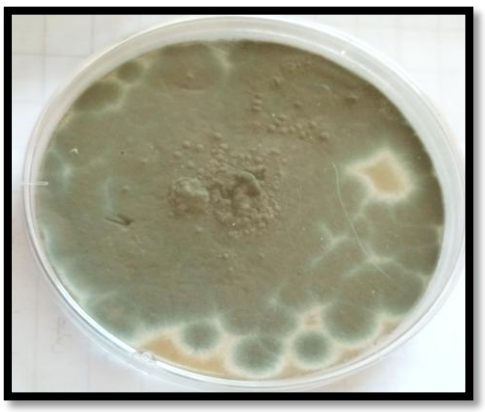

Figure (2) Macroscopically appearance of *Aspergillus fumigatus* on

CZA at 37°C for 7 days

Microscopic appearance

Under microscopic examination, the presence of green conidia forming chains originating from greenish phialides was observed in *Aspergillus fumigatus* colonies grown on Cazpek dextrose agar at 37°C for four or seven days. Additionally, the vesicles displayed a subclavate shape when stained with lactophenol cotton blue as in figure (3).

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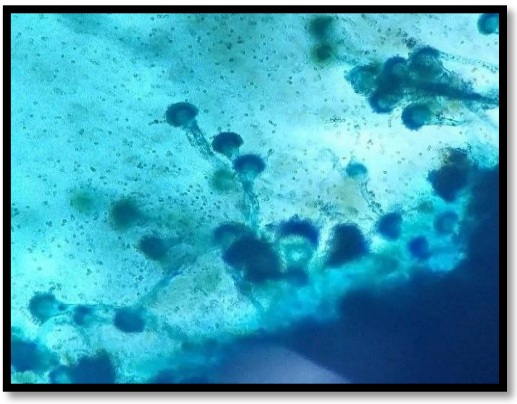

Figure (3): Microscopically appearance of *Aspergillus fumigatus* stained with Lacto phenol Cotton blue (X40).

Extraction and quantification of gliotoxin

The findings of this investigation revealed that Yeast Extract Broth (YEB) Which consist of 4% sucrose and 2% yeast extract dissolved in 1000 ml of distilled water and incubated at 37 °C for seven days served as an efficient medium for the rapid assessment of *Aspergillus fumigatus* growth and (GT) biosynthesis, as illustrated in Figure 4. Moreover, High-Performance Liquid Chromatography (HPLC) analysis confirmed that all thirty tested isolates produced detectable levels of GT, with the highest concentration reaching 23.823 µg \kg.

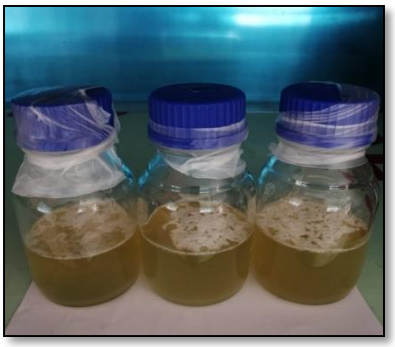

Figure (4) *Aspergillus fumigatus* growth on YEA after 7 days at 37°C for GT production

Production of Gliotoxin and Detection by HPLC.

The potential of *Aspergillus fumigatus* isolates to produce GT was evaluated using Yeast Extract Broth (YEB), and the toxin concentration was quantified through High-Performance Liquid Chromatography (HPLC) analysis. One representative *A. fumigatus* isolate was assessed for GT biosynthesis in YEB, showing a yield of 23.823 µg/kg as determined by HPLC. The results

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confirmed that the yeast extract broth served as an efficient medium for GT production, as illustrated in Figure 5.

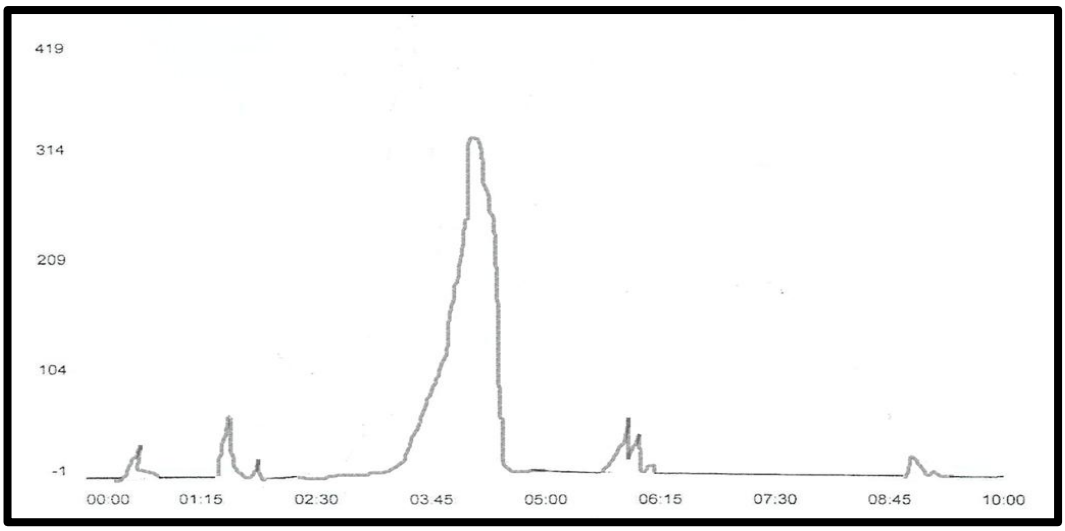

Figure (5): Detection of gliotoxin by HPLC

Discussion

The results of the current study revealed that Aspergillus *fumigatus* is one of the most common species of fungus that causes respiratory infections in cattle. Similar findings were also provided by Shan and Panjabi (2016), who cultured *A. fumigatus* on tracheal tissues and swab samples and also on bronchial mucosa, where its presence led to necrotic tracheobronchitis in a cow.

Bovine Respiratory Disease (BRD) is a microbial infection of lung parenchymas of beef and dairy cattle, which is highly morbid and fatal (Holman et al., 2015). The disease causes loss of animals, low feed intake and high costs of treatment which all adds to high economic losses amounting to USD 800-900 million in the United States alone annually (Chirase and Greene, 2001).

Pulmonary system aspergillosis is the dominant mycotic condition of the pulmonary system, related to *Aspergillus* spp., a widespread mold that inhabits indoors as well as outdoors (Kousha, 2011). *Aspergillus* infection in the clinic is often in the form of invasive pulmonary aspergillosis, one of the severe forms of the disease with a fatality rate of about 90%. The common species that may be isolated out of airways include: *A. fumigatus*, *A. niger*, *A. flavus*, and *A. terreus* in which the invasion may advance to aggressive aspergillosis or spread aspergillosis (Kosalec and Pepeljnjak, 2005).

Aspergillus fumigatus replicates asexually by the generation of spores. Therefore, conidial spores develop in abundance through germination process and are commonly ingested by hundreds of animals daily (Abdel-Gawad, 2021). Also *Rhizopus*, *Mucar*, and *Candida* species were

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predominantly infected immunocompromised hosts, resulting in respiratory and generalized infections mainly through spore breathing (Kontoyiannis *et al.*, 2006). According to Ozgun *et al*. (2012), all these isolates of fungi were widespread soil fungal saprophytic organisms that are important infections that may adhere to the surfaces, generate proteases and collagenases, and induce lesions that may spread to various organs or cause clinically invasive infections in the lungs.

These results of this study were almost similar to that described by (Hussein *et al*., 2017) who demonstrated that the yeast extract broth was the appropriate culture medium for the proliferation of *Aspergillus fumigatus* and GT production, examined by HPLC. While Nouri *et al*. (2015) noted that the HPLC analysis revealed GT production at 122.6 ppm in rice medium which is the appropriate culture medium for GT production from *A. fumigatus* isolates. Moreover, Sael *et al*. (2019) revealed that the HPLC is a better test for screening large numbers of GT concentration that produced from *A. fumigates* and is quick, easy, cheap and available.

According to Zhang *et al.* (2012), the High-Performance Liquid Chromatography (HPLC) method used for gliotoxin quantification offers several advantages, including high accuracy, reproducibility, and reduced analysis time. Under optimized analytical conditions, this method achieves a recovery rate of up to 99.22%, influenced by factors such as the type of column, column temperature, and mobile phase composition.

The *Aspergillus* genus synthesizes various toxic secondary metabolites (mycotoxins), including GT, among others (Kilch, 2009). The growth and mycotoxin production of fungal species are strongly influenced by environmental parameters. In particular, water activity (aW) and temperature represent the primary determinants governing fungal germination, hyphal development, and sporulation (Bellí *et al.*, 2004; Peña *et al.*, 2015). Moreover, the genetic variability among fungal strains, combined with environmental conditions, can significantly affect mycotoxin biosynthesis. Additionally, GT production has been shown to be oxygen-dependent, with the concentration of oxygen in the environment directly influencing its synthesis.

Conclusions:

According to this study, the most common fungus that causes respiratory infection in cows are *Aspergillus spp*; particulariy, *Aspergillus fumigatus* and the possibility of these fungi secreting toxins, including the GT and measuring by HPLC .

Recommendations:

We recommend An epidemiological study is required to map and detect the *Aspergillus fumigatus* in other animals in Iraq , also studies required to investigate the natural production of gliotoxin in local and imported animal feeds.

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Conflict of interest:

The authors declare no conflict of interest.

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Authors contribution:

All authors contributed effectively in this research

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