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Article

Cytotoxic effects of Gliotoxin extracted from *Candida albicans* isolated from patients with urinary tract infection

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Abstract: The findings of the toxicity test demonstrated the toxicity of exposure to the gliotoxin produced by *candida albicans*. Elevated medication levels were associated with an increased risk of blood deterioration. At a concentration of 200 µg/mL, the rate of degradation was 2.21%, and at a value of 100 µg/mL, it was 1.97%. The toxicity increased with prolonged contact with the fungus. And the Samples of gliotoxin were also tested on human lymphocytes to determine their cytotoxic effects. Using the methylthiazol tetrazolium (MTT) bioassay, the cells were subjected to four different doses of gliotoxin (100, 50, 25, and 12.5 µg/mL). Cell growth was shown to be concentration-dependent, with the sample exhibiting growth inhibition percentages at the corresponding concentrations of 33.82%, 10.16%, 5.7%, 0.0%, and 0.0%. The possible DNA damage caused by gliotoxin was evaluated by extracting DNA from lymphocytes and performing electrophoresis on a 1% agarose gel. The findings indicated that gliotoxin has the capacity to destroy or impair DNA. The study established a linear correlation among gliotoxin concentration, cell growth inhibition, and the degree of DNA damage in human lymphocytes. The study investigated the genotoxic effects of Gliotoxin (GT) on human lymphocytes, using single cell electrophoresis and a comet assay. Results showed significant DNA damage in these cells, highlighting GT's genotoxic impact. The comet assay revealed no significant differences in comet length between the control and concentration 50 µg, but the concentration 100 µg that shown significant changes in head diameter, head, tail, and tail content. These findings highlight GT adverse impact on DNA integrity.

Keywords: cytotoxicity assay; comet assay; FTIR; genotoxicity assay; HPLC; toxic test

1. Introduction

Urinary Tract Infections (UTIs) are common infections caused by bacteria or yeast entering the urinary tract [1]. They can affect both the lower and upper urinary tracts, presenting symptoms such as burning during urination and frequent urination of small volumes [2].

Candida albicans and non-*Candida albicans Candida* (NACA) species are integral constituents of the typical microbial flora present in the oral cavity, gastrointestinal system, and vagina of numerous healthy persons. They also reside in the external urethral opening of premenopausal women. Immune deficits can disturb the equilibrium between these yeasts and the host's normal flora, enabling commensal *Candida* to transform into opportunistic pathogens, resulting in candidal urinary tract infections [3,4].

The presence of *Candida* spp. in urine is termed candiduria, which can be asymptomatic or symptomatic. Symptomatic candiduria is associated with conditions such as cystitis, epididymorchitis, prostatitis, pyelonephritis, and renal candidiasis.

Asymptomatic candiduria is generally benign and not classified as a disease. *C. albicans* accounts for approximately 20% of nosocomial infections and is the primary cause of candiduria among over 200 *Candida* species [5].

Urinary tract candidiasis is the most prevalent nosocomial fungal infection worldwide, with *C. albicans* being the main etiological agent. However, there is a notable shift in the distribution of *Candida* species, accompanied by the emergence of antifungal-resistant strains. This research focuses on the uropathogenesis and dissemination of *C. albicans* and investigates the toxic effects of gliotoxin, a recognized virulence factor [6].

The present study aims to isolation and identification of *Candida spp***.** from patients with UTI and study the cytotoxic and genotoxic effects of gliotoxin.

2. Methods

2.1. Specimens' collection

Urine specimens were collected in a clean container. from patients suffering from urinary tract infections in Balad city in Salah al-Din Governorate from 1st July 2023 to 1st March 2024 in Balad General Hospital-Iraq.

All specimens were cultivated immediately on media for Bacteria and yeast (MacConkey and SDA agar plates). The plates were incubated aerobically at 37 °C according to the manufacturer's instructions [5]. Gram stain was performed for microscopic examination [4]. The identification of the genus *Candida* and its associated species was predicated on a number of tests, which encompass the following:

2.1.1. Cultural characteristics

The morphology, pigmentation, and texture of the *Candida* spp colonies cultivated on Sabouraud dextrose agar are analyzed using.

2.1.2. Microscopic test

The morphology of the yeast cells was analyzed microscopically following gram staining, at a 40 × magnification to assess their staining response, arrangement, and budding morphology [6,7].

2.1.3. Growth on chrom agar culture

The Chromo Agar plates were inoculated with yeast colonies using a sterile stick in a streaking method and thereafter incubated at 37 °C for 2 days, following which the growth and coloration of the colonies were evaluated [8,9].

2.1.4. Germ tube production test

In this procedure, little sterile test tubes containing 0.5 mL of human serum are infected with the yeast being studied. The tubes were incubated at 37 °C for 3–4 h, following which a drop of the liquid was placed on a clean glass slide, covered for microscopic examination, and seen at 40 × to identify the germination tube [10].

2.1.5. Gliotoxin extraction and analysis

Glytotoxin extracted from Selected *C. albicans*, was inoculated on a SD broth medium for 3 days at 37 °C. to allow biomass formation [11].

1) Gliotoxin was extracted from a selected strain of *C. albicans*.
2) The gliotoxin was inoculated into a SD broth medium and incubated for 3 days at 37 °C to allow biomass formation.
3) Gliotoxin was extracted twice using an electric homogenizer with 50 mL of chloroform.
4) The filtrate was then filtered through a Whatman No. 1 filter paper.
5) The collected filtrate after chloroform extraction was pooled and dried by evaporation.
6) The gliotoxin was stored at 4 °C [12].

After extraction, the concentration was determined by High Performance Liquid Chromatography (HPLC) and compared with standard gliotoxin. The separation occurred on a Shimadzu 10AV-LC liquid chromatography system equipped with a binary delivery pump model LC-10A Shimadzu. The eluted peaks were monitored by a UV-Vis 10A-SPD spectrophotometer.

The standard gliotoxin used was obtained from Sigma-Aldrich (ID: 24895384, Molecular Weight: 326.39 g/mol) [10].

The HPLC analysis was performed under the following conditions:

- Column: HCMA-BIO 1000 C-18, 3 μm particle size, 50 × 4.6 mm I.D;
- Mobile Phase: Methanol:Water (43:57, v/v) with 1% trichloroacetic acid (TCD);
- Detection: UV at 254 nm;
- Temperature: Ambient;
- Flow Rate: 1.0 mL/min;
- Injection Volume: 20 μL.

Fourier-transform infrared spectroscopy (FTIR) used for gliotoxin analysis to load the fungal crude extract and scan it between 400–4500 cm^{-1}. Based on the graph, various functional groups were deduced [13].

The toxicity of *Candida* was tested using a hemolysis experiment. The experiment involved mixing *Candida* with blood, adding physiological saline solution, and centrifuging at 3000 revolutions for 10 min. The spectrophotometer at a wavelength of 540 was used to measure the hemolysis rate. A positive control was added to the blood solution and measured using a spectrometer at a wavelength of 540. The rate of hemolysis was determined as 100% by diluting the blood with a volume 100 times greater than normal saline solution. The reading was represented by the symbol (A100H) and the equation was applied. The spectrometer readings were represented by symbols AA and AB.

The results determine using the following equation Hemolysis% = (AA − AB)/(A100%H − AB) × 100%.

AA *Candida*, blood and physiological saline, AB *Candida*, DMSO, and physiological saline, Blood and distilled water A100%H.

2.2. Human lymphocytes collection and preparation

Healthy 26-year-old male donor for Peripheral venous blood, 10 mL of blood were taken in 50 mL sterile tubes containing the anticoagulant EDTA. This blood was utilized for lymphocyte isolation. The human lymphocytes were extracted

according to the following [14,15]. The plates of human lymphocyte cell were then incubated in an incubator supplemented with 5% CO_2 at 37 °C for 24 h [15]. Following the incubation period, varying concentrations of gliotoxin were introduced to each well [16].

2.3. Cytotoxicity assay using methyl tetrazolium (MTT) dye and genotoxicity

The experiment employed 3-[4,5-dimethylthiazoyl]-2,5-diphenyltetrazolium bromide (MTT), which was prepared by diluting it in phosphate-buffered saline (PBS) to a concentration of 2 mg/mL and subsequently filtered through a 0.22 μm Millipore filter. A volume of 50 μL of MTT was added to each well of a microtiter plate containing 10^4 of human lymphocytes cells exposed to various doses of gliotoxin for 24 h. MTT-formazan crystals, generated only by live cells, were solubilized in 100 μl of dimethyl sulfoxide (DMSO) to assess the optical density at 620 nm with an ELISA reader. The inhibitory rate was subsequently determined [7]. In this study, also subjected control without (DMSO) only culture media and human lymphocyte was used for comparison with the other samples.

DNA was extracted from cells using a genomic DNA micro kit from the Promega Company [ID:pr3865ss] and follow all the steeps in the leaflet. The extracted DNA were electrophoresed on a 1% agarose gel using the optimum condition of electrophorese to show the results of genotoxicity [16].

2.4. Comet assay

Using a single cell electrophoresis (Comet assay) method, the DNA damage of cultured lymphocytes was evaluated for three concentrations of Gliotoxin the steeps stated by mixing low melting agarose (LM agarose) with cultivated cells, coating a Comet slide, and submerging it in lysis solution for 30–60 min. The slide was then submerged in an alkaline solution for 20–30 min. The Comet assay electrophoresis was performed, followed by submerging in 70% ethanol and deionized distilled water. The slide was then stained with 100 μL of SYBR for 30 min. After drying, the slide was inspected under a fluorescence microscope with a 40 × magnification. A Comet score was established by analyzing 50 randomly chosen cells from each sample, taking into account the extent of DNA damage [17].

2.5. Statistical analysis

The results analysis statistically using SPSS version 22, significant differences between means were assessed using one-way analysis of variance (ANOVA), followed by the Duncan test. The level of significance was set at $p < 0.05$ [18].

3. Results and discussion

Out of 120 specimens, 8 samples tested positive for *Candida* sp. the forming colonies manifested on Sabouraud dextrose agar (SDA) as white to cream-colored, smooth, round colonies, similar with the characteristics of *Candida* spp. Colonies have a creamy appearance when cultivated on the specified medium to facilitate growth conditions. The isolated species had a favorable interaction with the

chromium dye, exhibiting cell shapes ranging from oval to spherical, or oval to longitudinal, or cylindrical. This finding aligns with the results reported by Boon and his team [19]. The *Candida* cells have a blue coloration due to the presence of a peptidoglycan layer in their cell wall [20]. The *Candida* spp. were cultivated on ChromAgar medium for 24–48 h at 37 ℃. The findings indicated that each species have a distinct color. Four samples had a green hue attributed to *C. albicans*, while three samples displayed a light green coloration linked to C. glabrata, characterized by cream to white smooth colonies. The last isolates were C. krusei, which presented a purple, fuzzy appearance [21].

Detection of the gliotoxin by Fourier Transform Infrared FTIR and HPLC FTIR spectrographic analysis is used to determine the vibration frequencies of the bonds in Gliotoxin. The band values in the infrared radiation region are attributed to the chemical functional group of the molecule as show in **Figure 1**. The O-H bond, composed of alcohol, is responsible for absorption between 3549.02–3415.93 cm^{-1}. Absorptions from 2096.62 cm^{-1} are due to C≡C bands, while absorptions from 1637.56–1618-28 cm^{-1} are due to N–H bands. Absorptions from 1521.84 cm^{-1} are due to N–O bands, absorptions from 1406.11 cm^{-1} are due to C–H bands, absorptions from 1136.07 cm^{-1} are due to C–N bands, absorptions from 995.27 cm^{-1} are due to C=C bands, and absorptions from 634.58–621.08 cm^{-1} are due to C–Br bands.

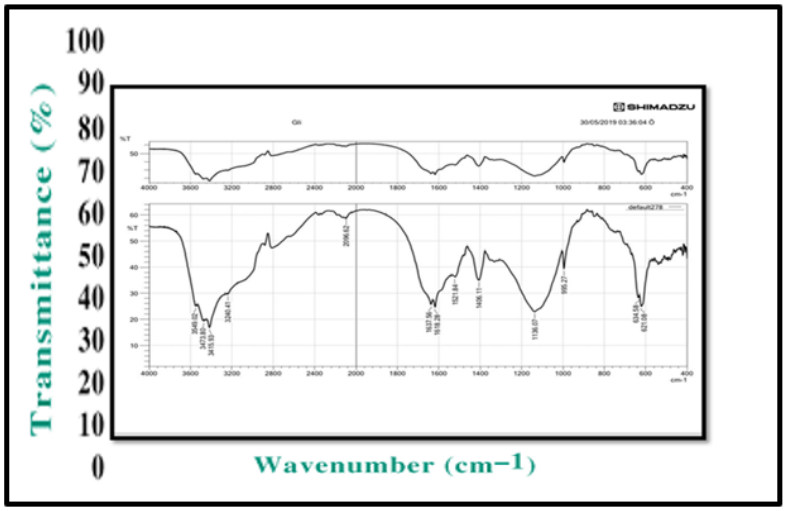
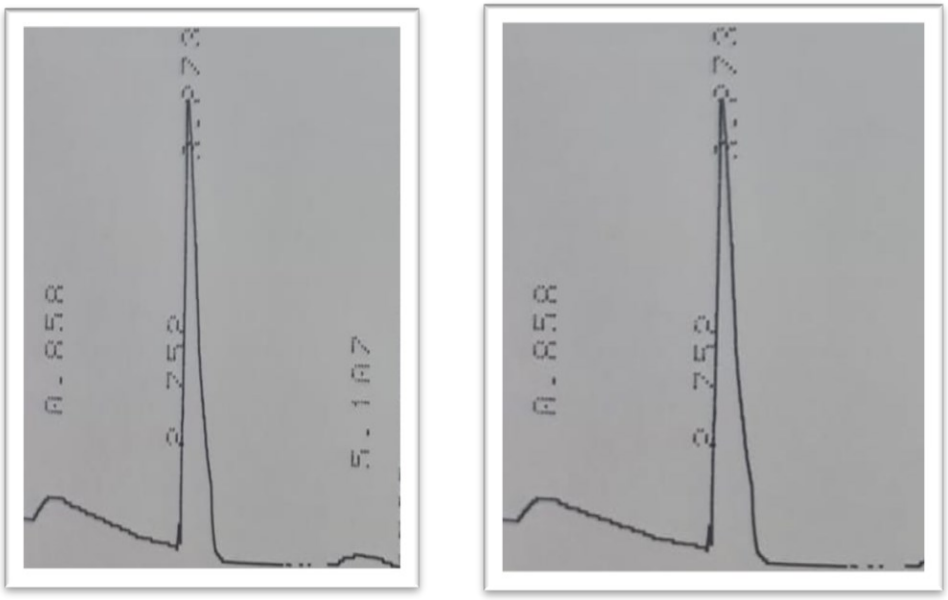


Figure 1. Infrared analysis of gliotoxin extracted from clinical *candida albicans* isolated from patients with urinary tract infection.

Gliotoxin was identified in certain strains of *Candida* albicans using high-performance liquid chromatography (HPLC). High performance liquid chromatography (HPLC) is a crucial qualitative and quantitative method, often employed for the analysis of pharmaceutical and biological specimens. It is the most adaptable, secure, reliable, and rapid chromatographic technology for the quality control of components [22].

The results in **Figure 2** showed that high gliotoxin production on SD broth medium gave positive result by HPLC, concentration of gliotoxin (10 μg/gm). The standard gliotoxin were separated on HPLC (High Performance Liquid

Chromatographic) column under the optimum separation condition, Column: HCMA-BIO 1000 C-18,3 μm particle size (50 × 4.6 mm I.D) column, Mobile phase: Methanol: water (43:57, v/v).1% trichloro acetic acid (TCD).



(A) **(B)**

Figure 2. Detection of gliotoxin extracted from clinical *Candida albicans* isolated from patients with urinary tract infection, **(A)** standard gliotoxin; **(B)** extracted gliotoxin.

SD broth medium, alongside conventional gliotoxin conditions, utilizing HPLC analysis; gliotoxin retention period is around 4.8 min. The mobile phase consisted of methanol and water at a ratio of 43:57, with a flow rate of 2.0 mL/min.

3.1. Toxicity test of *Candida albicans* test

In these experiments, the toxicity obtained indicate the toxicity of exposure to *C. albicans*. The higher the concentration of the substance, the greater the risk of blood degradation. At a concentration of 200 μg/mL, the degradation rate was 2.21%, and at a concentration of 100 μg/mL, the degradation rate was 1.97%. Toxicity increases with increased exposure to the *C. Albicans* as show in **Table 1**. The rate of hemolysis was determined as 100% by diluting the blood with a volume 100 times greater

$$\text{toxicity\%} = (AA - AB)(A100\%H - AB) \times 100\%$$

where each represents

AA *Candida*, blood and physiological saline;

B *Candida*, DMSO, and physiological saline;

Blood and distilled water A100%H;

$$\text{Hemolysis\%}(200\ \mu g/mL) = 0.127 - 0.062/3.000 - 0.062 \times 100\%$$

$$\text{Hemolysis\%}\ (100\ \mu g/mL) = 0.118 - 0.060/3.000 - 0.060 \times 100\%$$

Table 1. Toxic effect of clinical *Candida albicans* isolated from patients with urinary tract infection.

Sample	(0.1) g/mL	(0.2) g/mL
AA	0.118	0.127
AB	0.060	0.062
H	3.000	3.000
Hemolysis%	1.97%	2.21%

P value ≤ 0.05

3.2. Cytotoxicity effects of gliotoxin on human lymphocytes

Results in (**Table 2**) showed that growth inhibition of human lymphocyte was increased gradually with the increase of gliotoxin concentration when compared with the negative control (the same cell without any treatment). Gliotoxin has significant differences of cytotoxic effect on human lymphocyte $P < 0.05$, 33.82%, 10.16% and 5.7% growth inhibition was show at concentrations 10, 5, and 2.5 ng/mL respectively. The growth inhibition percentages were determined at various concentrations, demonstrating that the rate of growth inhibition was dependent on the concentration of gliotoxin (100, 50, 25, 12.5 and 6 µg/mL). A fungal toxin known as gliotoxin has the ability to stop angiogenesis, prevent NF-κB activation, and cause apoptosis. Because it can bind and inactivate proteins by sulfide: thiol exchange, which results in the formation of reactive oxygen species (ROS) through redox cycling, its intrinsic disulfide bridge is essential to its cytotoxic activity [23].

Table 2. Cytotoxicity effect of different concentrations of extracted gliotoxin from clinical *Candida albicans* isolated from patients with urinary tract infection on human lymphocyte after 24 hr.

Gliotoxin Concentration	Growth Inhibition %
100 µg	33.82% ± 20.76
50 µg	10.16% ± 3.87
25 µg	5.7% ± 8.00
12.5 µg	0.0% ± 0.00
6 µg	0.0% ± 0.00

P value ≤ 0.05.

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay to assess cell viability and cytotoxicity. Gliotoxin has been reported to induce apoptosis by elevating the concentration of C-AMP and lead to morphological changes in cells, including loss of adherence. Other studies have investigated the immunosuppressive effects of gliotoxin using lymphoblastogenesis and cytotoxicity assays [24]. Results showed that concentrations of gliotoxin below 25 µg had little or no response or stimulation, while higher concentrations led to cell death [4].

Gliotoxin demonstrated promise as an immunosuppressive agent to reduce allograft rejection in experiments conducted using human lymphocytes. It showed co-mitogenic action and lowered intracellular C-AMP levels at low dosages, causing apoptosis and raising intracellular C-AMP levels at greater concentrations. Modified

levels of C-AMP may mediate the effects of gliotoxin on cell activation and apoptotic induction. Overall, the research suggests that gliotoxin has a concentration-dependent cytotoxic effect on lymphocytes, inducing apoptosis and affecting cell viability [25].

3.3. Genotoxicity effects of gliotoxin on human lymphocytes

DNA was isolated from human lymphocytes treated with several dosages of gliotoxin: 100, 50, 25, and 12.5 µg/mL, and subsequently examined by electrophoresis on a 1% agarose gel. Gliotoxin shown the capacity to degrade or destroy DNA. The results indicated a progressive increase in DNA damage corresponding to the rising concentration of gliotoxin (**Figure 3**). Gliotoxin exhibits biological properties that facilitate redox cycling, leading to the generation of oxygen free radicals that cause oxidative damage to isolated DNA in vitro. This toxicity affects the immune system, resulting in apoptotic cell death in thymocytes, peripheral lymphocytes, macrophages, spleen cells, and others. This process is characterized by DNA breakage and the formation of adducts [26].

The genotoxic action of gliotoxin demonstrated a dose-dependent increase in DNA damage in human lymphocytes. Additionally, the DNA-reactivity of gliotoxin has been examined in several in vitro experiments [27], which indicate that the reactive oxygen species generated by gliotoxin can induce DNA damage. DNA damage in cells exposed to gliotoxin was observed at 24 h, particularly at elevated concentrations of gliotoxin (50–100 µg) [28].

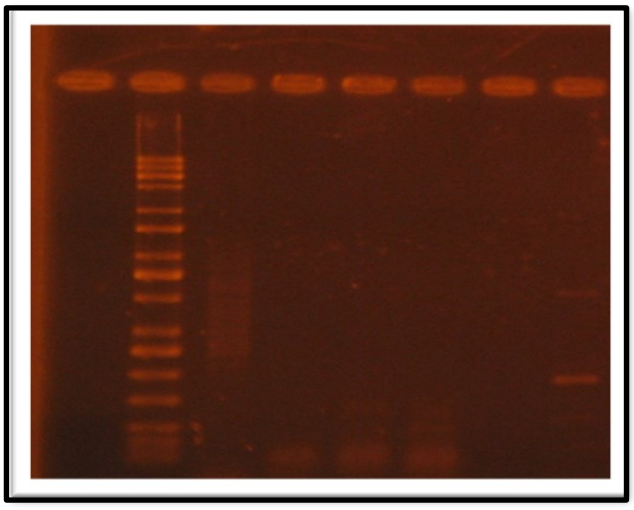


Figure 3. Agarose gel electrophoresis of the total genomic DNA for human lymphocyte treated with gliotoxin fragments were fractionated by electrophoresis on a 1% agarose gel staining with ethidium bromide.

3.4. Comet assay for gliotoxin

The genotoxicity of gliotoxin was verified using single cell electrophoresis of human lymphocytes treated with varying concentrations of gliotoxin (100−50 µg/mL). The analysis revealed a substantial DNA damage in these cells, as shown in (**Table 3**).

Table 3. Comet assay for gliotoxin extracted from clinical *Candida albicans* isolated from patients with urinary tract infection.

Comet parameters	Control Mean ±SD	(100) µg/mL Mean ± SD	(50)µg/mL Mean ± SD	*p*-value
Comet height	81.04 ± 31.88	81.51 ± 15.96	73.79 ± 19.73	0.344 ns
Head diameter	78.81 ± 32.85	61.56 ± 17.19	51.59 ± 20.18	0.003**
DNA in head	93.17 ± 7.334	99.19 ± 5.287	10.59 ± 9.875	0.001**
Tail length	10.46 ± 3.980	12.590 ± 1.523	9.641 ± 4.675	0.588 ns
DNA in tail	6.832 ± 1.438	4.806 ± 0.846	4.806 ± 1.581	0.001**

** Highly significant ≤ 0.01

The discovery emphasized the genotoxic impact of GT. The comet test was employed to identify breaks in the DNA of apoptotic cells, whether they were single or double stranded [29]. as show in **Figures 4–6**.

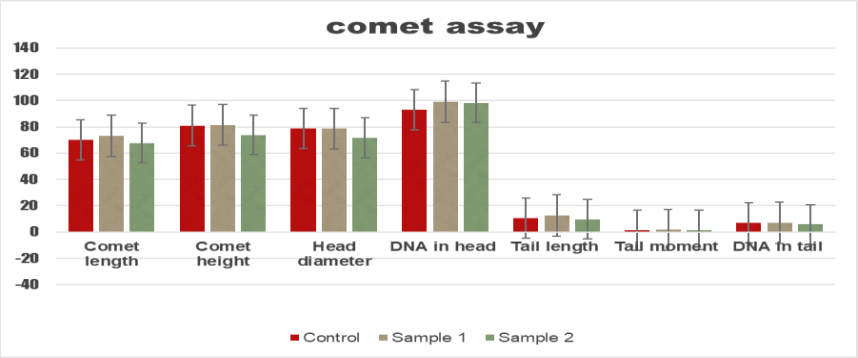


Figure 4. Diagram of comet assay result of gliotoxin extracted from clinical *Candida albicans* isolated from patients with urinary tract infection.

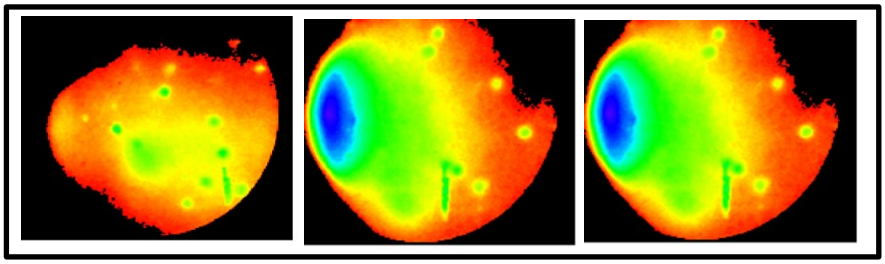


Figure 5. Concentration 100µg/mLcell electrophoresis of cultured human lymphocytes after treated with gliotoxin concentration 100 µg/mL.
Sample 1, significant differences in head diameter, head and tail DNA.

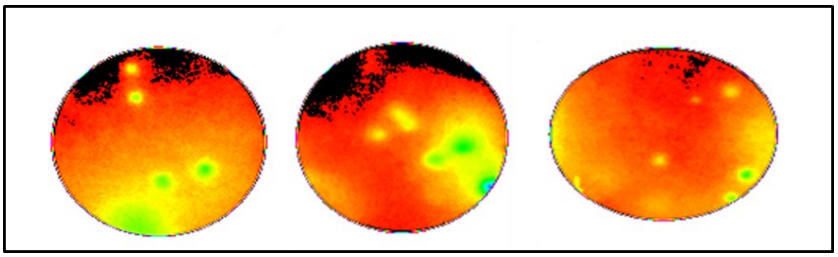


Figure 6. Concentration 50 µg/mL cell electrophoresis of cultured human lymphocytes after treated with gliotoxin concentration 50 µg/mL.
No significant difference in comet length and tail length between the control and sample 2, but significant differences in head diameter, head and tail DNA.

The result of the comet assay is no significant variation in comet length between the control group and sample 1 ($p > 0.05$), But there are significant changes in head diameter, DNA content in the head, tail length, and DNA content in the tail between the control group and sample 1 ($p < 0.05$).

There is no statistically significant difference in comet length, comet height, tail length, and tail moment between the control group and sample 2 ($p > 0.05$). There are significant changes in head diameter, DNA content in the head, and DNA content in the tail between the control group and sample 2 ($p < 0.05$).

The results of this study aligned with the findings of previous studies conducted to assess DNA damage in lymphocytic cells using the Comet Assay [30,31].

The clinical *Aspergillus fumigatus* which the main producer of gliotoxin is significant in the postallogeneic SCT context [9]. Given that invasive aspergillosis affects approximately 4% to 10% of SCT recipients and has overall mortality rates ranging from 30% to 80%, it is imperative to identify ways to battle this disease [2,8]. The identification of the mechanisms via which AF causes pathogenesis may help design more effective preventative measures and treatment plans [9]. The study's findings show that very rare amounts of AF-specific T lymphocytes were found in the peripheral blood of healthy donors. Crucially, we also showed that the inhibition of functional T-cell responses in vitro was linked to gliotoxin [20].

The results reported detectable proliferative responses using a comparable AF-derived antigen preparation in the majority of donors studied, are different from ours since we used specific T-cell responses [15]. We assessed cytokine production at the single-cell level using flow cytometry, cytokine levels in cell-free supernatants following PBMC stimulation [22]. Although our earlier research and other studies have confirmed the use of CFC-based assays as a precise and clinically significant method to identify the frequencies of human T cells recognizing CMV, HIV, and other pertinent infections, it is probable that the method we employed here is less sensitive [19].

4. Conclusions

We concluded from the study that gliotoxin, exhibits a greater cytotoxic impact and greater DNA damage, the effect of gliotoxin on comet assay show dose-dependent manner.

Author contributions: Conceptualization, YQH and BID; methodology, YQH and BID; software, YQH and BID, original draft preparation, YQH and BID; writing, YQH and BID. All authors have read and agreed to the published version of the manuscript.

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Ethical approval: The study was conducted in accordance with the Declaration of Helsinki, and approved by the Institutional Review Board OF College of Applied science (protocol code ID:23302023 and date of 14/10/2023).

Availability of data and materials: The data underlying this article are available in the article and in its references. The derived data generated in this research will be shared on reasonable request to the corresponding author.

Conflict of interest: The authors declare no conflict of interest.

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