Cytotoxic effect of Gliotoxin from Candida spp. isolated from clinical sources against cancer and normal cell lines

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Abstract

Background: Invasive fungal infections have become more common during the past two decades. Candida species are the most common human fungal infections. Internal injuries characterize these infections because of virulence factors, such as gliotoxin, which is a fungal toxin that is thought to be antibacterial, antifungal, and antiviral.

Objectives: To test the ability of Candida species obtained from clinical sources to produce gliotoxin as a virulence factor and investigate its cytotoxicity effects against some selected cell lines.

Materials and Methods: One hundred and ten clinical isolates of Candida species were obtained from patients attending hospitals in Baghdad from September 2021 to March 2022. They were diagnosed and characterized by routine laboratory methods and cultures. The capability of Candida isolates to secrete the gliotoxin was tested and measured by analytical methods. The cytotoxicity of produced gliotoxin was applied against normal and cancer cell lines.

Results: The 110 yeast isolates were diagnosed and identified as follows: Candida albicans, Candida tropicalis, Candida parapsilosis, Candida glabrata, Candida Krusei, Candida kefyr, Candida lusitaniae, Candida rugosa. Twenty-eight Candida isolates showed gliotoxin production. The cytotoxicity effects of gliotoxin were reported against lymphocytes and AMGM and AMJ13 cell lines in different concentrations. The highest cytotoxic effect was noticed in the concentration of 400 µg/mL of gliotoxin.

Conclusion: The results indicated that the pathogenicity of Candida was distributed among all ages, both sexes, and several types of sources of clinical isolates. Gliotoxin had an effect on normal and cancer cells.

Keywords: Candida albicans, gliotoxin, HPLC, cytotoxicity.

Introduction:

It is estimated that fungus-related infections cause more than 1.5 million fatalities annually, with a burden on the planet of over one billion (1). Despite this, the problem of fungal pathogenicity has received little attention (2). Invasive fungal infections have become more common during the past two decades (1). Additionally, the prevalence of invasive fungal diseases is rising along with the number of susceptible at-risk patients, such as those who are immunosuppressed due to transplants, corticosteroid therapies, AIDS, autoimmune diseases, cancer, or patients undergoing major surgery, among other risk factors. (1, 3) The human-associated commensal and polymorphism fungi, such as the Candida species, are the cause of the most common human fungal infections (3, 4). They do not spread illnesses to healthy humans but are regarded as opportunistic pathogens that only result in infections under favorable settings and in certain clinical situations (5). These infections are characterized by internal injuries such as those to the mouth, gastrointestinal tract, urinary tract, and genital tracts because of its virulence factors. (6) There are roughly 150 species in the genus Candida. The top seven known disease-causing species are seven of them: C. albicans, C. parapsilosis, C. tropicalis, C. krusei, C. glabrata, C. kefyr, and C. guilliermondii (7). A mycotoxin is a toxic secondary metabolite that is created by members of the fungi kingdom and can harm or kill both humans and other animals (8). A severe mycotoxin known as gliotoxin (GT) is generated by species of fungus from several genera, including Candida yeasts (8). (GT) is an epipolythiodioxopiperazine (ETP), which is a kind of fungal toxin that is thought to be antibacterial, antifungal, and antiviral. It has a disulfide bridge across the piperazine ring, which seems to be involved in the toxic actions. Yeasts known as Candida species obstruct a variety of human and animal commensal microflora locations (9). GT has been demonstrated to inhibit a variety of different mechanisms at the cellular level in human activation of transcription factor NF-B, including ion Ca2 release from mitochondria, diversity and response to stimuli in T and B cells, chymotrypsin of the 20S proteosome inhibition of various

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activities, inhibitor of ferranylgranal transferase and geranylgranal transferase, and others (8). The mode of action of GT remains unclear. Therefore, the main goal of our search was to further evaluate GT in vitro activity, as well as its putative mode of action, in normal cell. It also aimed to investigate the influence of GT on breast cancer and brain cancer cell lines.

Materials and methods
Collection and identification of Candida isolates
One hundred and ten Candida isolates were sampled from 430 clinical samples of individuals thought to have candidiasis from various sources of the body including swabs, blood, urine, ear, and skin of people who attended many hospitals in Baghdad City, during the period from September 2021 to March 2022. These samples were not duplicates, and with routine steps, all yeast isolates were cultured and grown on SDA agar (Oxoid). The yeast isolates were tested directly using the compound microscope. Candida spp. yeasts were identified according to genus and species using phenotypic characteristics by Germ tube formation in fresh human serum, according to Sheppard et al (10), Growth at 45°C (11), Chlamydomspore formation on specific medium corn meal agar (Himedia-India) with Tween 80 (12). To distinguish between the various species of Candida, the CHROMagar medium (Himedia-India) was prepared in accordance with the manufacturer’s directions and placed into sterile petri plates. The isolated Candida strains were parallel injected onto CHROMagar and cultured in petri plates for 24 hours at 37°C based on the instructions of the Manufacture Company. The morphological features and pigmentation of all clinical isolates of yeast were studied and determined (13). For more conformation, Candida isolates were characterized and identified by the VITEK2 system.

Production of Secondary metabolites GT using Candida spp. isolates
All the obtained clinical strains of Candida spp. were subjected to produce GT. Firstly, the strains were cultured on SDA for 24 hours at 37 °C, after that, the production of GT was accomplished according to Kuphal et al (14) with some modifications. The colonies of Candida spp. were inoculated on sterile Roux culture bottles (250 mL) 100mL of RPMI 1640 and the volume adding 5% (v/v) of fetal calf serum (Sigma/Germany). Then they were incubated for 7 days at a temperature of 37 °C (130 rpm) in a rotating shaker incubator 5% CO2. To separate the cells from the mixture, they were placed in a centrifuge at 5,500 rpm for 30 min. The mixture was diluted with chloroform (3X) and filtered by means of filter paper that is placed in a funnel and anhydrous sodium sulfate is placed in it. An evaporator was used to evaporate the chloroform portion. The remaining was dissolved in 200 µl methanol. After that, it was filtered using a Millipore 0.45 µm filter unit.

Characterization of GT using Thin Layer Chromatography
TLC technique was used to investigate and characterize the produced GT in a broth culture medium. 10 microliters of each extract in methanol and of a 1 mg ml⁻¹ GT standard in methanol were spotted onto a Silica gel 60 plate (Merck, Damstadt, Germany) approximately 3 cm from the bottom edge. The plates were developed for 10 cm in an in-lined tank with a solvent system composed of toluene, formic acid, and ethyl acetate (5:1.4). The TLC plates were dried at 25-30°C. Then, the TLC plates were tested using fluorescent light under long UV light 365 nm and short UV light 254 nm (15), and \( R_f \) value were calculated using the bellow equation:

\[ R_f \text{ value}= \text{Distance spot move/ Distance solvent move} \]

Detection of GT by High Performance Liquid Chromatography analysis
The chloroform extract in methanol containing GT was diluted 1:5 and 20 µl of each fraction was injected into the HPLC system. The HPLC system under consideration included a Shimadzu CRI-B data processor, a water model 510 pump fitted with a U6K septum less injector, and a 10 cm x 4.6 mm RP analytical column (10 micrometers) with a 3 cm guard column and 10 1 RP -18 packing. Methanol and water made up the mobile phase, with a flow rate of 2 ml/min. To measure retention time (RT) and relative peak area, injections of 20, 40, 60, and 80 ng of standard GT dissolved in the mobile phase were made. The association between peak area amounts (ng) administered and sample concentration of GT was established using a standard curve (16).

Preparation of GT concentrations
In order to prepare concentrations of 100, 200, 300, and 400 µg/ml in whole culture medium (RPMI-1640 medium added with 10% fetal calf serum, contained a solution of streptomycin 100 µg/ml and penicillin 100 units/ml), GT extract was dissolved in methanol at 1 ng/ml (17).

Cytotoxic effect of GT against normal and cancer cell lines.
Preparation of human lymphocytes
Healthy 28-year-old male donors who had not taken any medications altering lymphocyte functions for at least two weeks prior to the blood collection for this study. They have given their informed consent before having their blood samples drawn. To prevent lymphocyte activation loss, the venous blood sample was taken from the antecubital vein and anticoagulated with trisodium citrate (0.0108 M) within an hour of collection. Differential centrifugation at 200g for 10 minutes at room temperature was used to create lymphocytes (18).
Cytotoxicty of CT Using the 3-(4,5-dimethylthiazol-2-y)-2,5-diphenyltetrazolium bromide (MTT) assay

Statistical analysis of the collected data was assessed via the SPSS 15.0 program. The independent t-test was used to determine the differences between mean values. A probability of p < 0.05 and p < 0.01 was considered statistically significant and highly significant, respectively (22).

Table 1: Distribution of obtained isolates according age and gender of patients

<table>
<thead>
<tr>
<th>Age (Years)</th>
<th>Gender</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-10</td>
<td>Male</td>
<td>51</td>
</tr>
<tr>
<td>0-10</td>
<td>Female</td>
<td>110</td>
</tr>
<tr>
<td>11-20</td>
<td>Male</td>
<td>100</td>
</tr>
<tr>
<td>11-20</td>
<td>Female</td>
<td>210</td>
</tr>
<tr>
<td>21-30</td>
<td>Male</td>
<td>150</td>
</tr>
<tr>
<td>21-30</td>
<td>Female</td>
<td>300</td>
</tr>
<tr>
<td>31-40</td>
<td>Male</td>
<td>200</td>
</tr>
<tr>
<td>31-40</td>
<td>Female</td>
<td>400</td>
</tr>
<tr>
<td>41-50</td>
<td>Male</td>
<td>150</td>
</tr>
<tr>
<td>41-50</td>
<td>Female</td>
<td>300</td>
</tr>
<tr>
<td>51-60</td>
<td>Male</td>
<td>100</td>
</tr>
<tr>
<td>51-60</td>
<td>Female</td>
<td>200</td>
</tr>
<tr>
<td>&gt;60</td>
<td>Male</td>
<td>50</td>
</tr>
<tr>
<td>&gt;60</td>
<td>Female</td>
<td>100</td>
</tr>
</tbody>
</table>

Table 2: Distribution of Candida spp. isolates based on age of patients

<table>
<thead>
<tr>
<th>Age Group</th>
<th>C. albicans</th>
<th>Other Candida</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infant</td>
<td>50</td>
<td>100</td>
<td>150</td>
</tr>
<tr>
<td>Child</td>
<td>150</td>
<td>300</td>
<td>450</td>
</tr>
<tr>
<td>Adult</td>
<td>150</td>
<td>300</td>
<td>450</td>
</tr>
<tr>
<td>Elder</td>
<td>50</td>
<td>100</td>
<td>150</td>
</tr>
</tbody>
</table>

The distribution of Candida isolates (C. albicans and other Candida spp.) by age groups is shown in Table 2. The highest percentage of C. albicans isolates was in the infant group (33.3%), followed by the adult group (28.9%). Other Candida spp. were more prevalent in the child group (22.2%), and in the elderly group (20%). The multiple cells in the table with identical values were tested for statistical significance using the Chi-Square test (p < 0.05).
Detection and Characterization of Secondary metabolites GT using TLC and HPLC analysis

Our results of TLC indicate that the Rf values of extracted GT from investigated isolates was 0.38 as shown in the figure (1). The data obtained from the HPLC analysis of extracted GT and standard revealed that the retention time of Candida isolates was 5.18, for standard GT, while the retention time of Candida isolates was 5.08 as shown in the figure (2).

Cytotoxic effect of GT against lymphocytes and cancer cell lines

The percentage cytotoxicity increased with increasing concentrations, with the highest concentration (400 µg/mL) of gliotoxin showing the highest inhibition of 85%, while the lowest concentrations showed 71%, 17%, and 12% of inhibition, respectively, when compared with the negative control as shown in the figure (3).

Cytotoxic effect of GT against AMGM and AMJ13 cell lines

The results indicate that the number of viable cells decreased gradually with increasing concentrations of GT as shown in the Figures (4, 5). These findings demonstrate that GT has general cytotoxic effects through the apoptosis among AMGM and AMJ13 cell lines.

Figure (1): Gliotoxin detection of extracted GT from C. albicans isolates using a TLC plate (numbers 3-7) compared to standard GT (S)

Figure (2): Detection of GT in the extracted culture supernatant, analysed using HPLC results. A- GT had a retention time of the main peak of 5.18 min as reported by injection of a GT standard. B- GT had a retention time of the main peak of 5.18 min as reported by injection of an extracted culture supernatant GT of C. albicans isolates.

Cytotoxic effect of GT against lymphocytes

Figure (3): Cytotoxicity effects of different concentrations (100-400 µg/mL) of gliotoxin on lymphocytes for 24 hours on MTT test.

Figure (4): Cytotoxicity effect of different concentrations (100-400 µg/mL) of gliotoxin on AMGM cells for 48 hours by crystal violate test.

Figure (5): Cytotoxicity effect of different concentrations (100-400 µg/mL) of gliotoxin on AMJ13 cells for 48 hours by crystal violate test.
Discussion

Candida spp. has increasingly emerged as principal pathogen of opportunistic infections in healthcare settings. Therefore, early isolation, speciation and antifungal susceptibility testing are essential for the clinicians to choose the best therapeutic approach for the patients to reduce morbidity and mortality (26). According to the patients' ages, the highest percentage of candidemia caused by Candida spp. was detected in newborns and young children. The types of Candida isolates collected during this study in low percentage were non-albicans, compared to C. albicans isolates, which isolated in a large number and high percentage. This finding is consistence with study of Nandini et al (27). Our results revealed that the infection by Candida spp. has increased with the age group 21-50 and decreased with increasing age of patients. However, research from some countries such Iran, India and South Korea supported our results (28, 29, 30, 31). The primary factor in the adult age group associated with infection by candidiasis may be that these groups participate more in social and occupational settings where there is a high risk of infected by Candida (31). Other studies suggested that individuals under the age of 20 are more susceptible to candidiasis. This also agrees with the results of the current study, as the infection rate was higher. Similar to this, the percentage of C. albicans isolates was 65.5% from cases of candidiasis in our investigation may be due to the isolates of C. albicans can grow in a variety of morphological forms, include unicellular budding, hyphae, pseudohyphae, that increase its virulence and invading tissue of hosts (33). In addition, underlying illnesses, immunosuppressive conditions, antibiotic therapy, and variations in the body internal environment are the reasons why the once commensal C. albicans turned into a genuine pathogen (32).

Our result of TLC assay showed that the Rf value of extracted gliotoxin from investigated isolates was 0.38, in consistence to the results of Jayalakshmi et al., 2021 (34), who reported that the Rf values of extracted and investigated GT from cultivated Trichoderma isolates were 0.44. Our findings of HPLC data analysis showed that the extracted GT from clinical strains of Candida species, notably C. albicans, C. tropicalis, and C. glabrata produced highest levels of GT in a liquid culture medium compared with the other tested isolates. The obtained data and results are in consistence with the results of Shah and Larsen's study (35), where GT standards eluted as a single, symmetrical peak in 5 minutes. The existence of several types of fungi and yeasts, not implicated in causes of various diseases suggests that the C. albicans and A. fumigatus may secrete many types of virulent factors that are very significant in supporting the pathogenic yeasts and fungi to invade and colonize host cells and tissue. Many reports and studies reported a significant function for GT in the pathogenicity of C. albicans, as this toxin is produced by a wide range of pathogenic strains of A. fumigatus and C. albicans. Alveolar macrophages, neutrophils, ciliary cells and lymphocytes are the general cellular constituents of the human and animal immune system responsible for the defense against A. fumigatus and C. albicans (36, 37). Mullbacher and Eichner, 1984 reported that the phagocytosis process inhibited by GT produced by A. fumigatus, whereas other scientists reported that GT is responsible for alterations in morphological charters of macrophage (38). GT has also been reported to induce apoptosis in immune cells and other cancer cell lines such as HT1080 cell (39), NR8383 (40) cell lines. Using GT as an anticancer toxin was first reported and hypothesized in 1947, in 2004 when it was shown to be very effective against six types of breast cancer cell lines, with IC50 levels ranging from (38 to 985 nM). GT was shown to be an effective inhibitor to prostate cancer (PC-3) and human leukemia (U-937) cell lines in 2012 and the IC50 levels were (0.2 and 0.4 μM), respectively (41). The mode of action of GT to induce apoptosis and proliferation through the elevation of the cyclic adenosine monophosphate (cAMP) concentrations (42). cAMP is a well-known and effective immune system regulator of innate and adaptive immune cell activity (43). Recently, more studies demonstrated that GT was used as an anticancer agent against specific cell line called (U87) through directly bonding to Pyruvate kinase isozymes M2 (PKM2) by the surface plasmon resonance assay (SPR) and cellular thermal shift assay (CETSA). This means that PKM2 is the active site of GT (44). In Iraq, Dheeb et al (2013) demonstrated that GT in concentrations ranged from (0.12 to 125 ng/mL) and has anti-tumor efficacy toward the specific type of cancer cell line called (human hepatocellular carcinoma, HepG2) cell line. The anti-tumor efficacy increased with increasing GT amount. GT has also been demonstrated to cause proliferation and apoptosis in lymphocytes and macrophages (45, 46). The best concentration of GT on apoptosis clearly happens with a concentration of 1μg/ml (47). To investigate the activity and cytotoxic effect of GT on NF-B signaling, the expression of NF-B and the nuclear translocation kinetics of the p65 subunit were studied after GT administration (39). Treatment with GT stopped NF-B from activating. High amounts of (NF-B-p65) were present in the nucleus fragment of control HT1080 cells that had not been treated. Nevertheless, exposure to GT may cause a decrease in the levels of nuclear-localized NF-B-p65 (39).
Conclusion
The results indicated that the pathogenicity of Candida was distributed among all ages, both sexes, and several types of sources of clinical isolates. Gliotoxin had an effect on normal and cancer cells.

Author Contribution
All the authors made a substantial, direct, and intellectual contribution to the search and approved it for publication.

Authors’ Declarations:
Conflicts of Interest: None.-
We hereby confirm that all the Figures and Tables in the manuscript are ours. Besides, the Figures and images, which are not ours, have been given permission for re-publication attached with the manuscript.-Authors sign on ethical consideration’s approval-Ethical Clearance: The project was approved by the local ethical committee Welfare Teaching Hospital Baghdad according to the code number (293002 on 02/11/2021).

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