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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 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. kefir, 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 protosome inhibition of various

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activities, inhibitor of ferranylgranyl transferase and geranylgranyl 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), Chlamydospore 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 Kupfahl 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% CO_2. 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 25-30ºC. 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 value=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 l 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 mg/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).

Cytotoxic efficacy of GT using the 3-[4,5-dimethylthiazoyl]-2, 5- diphenyltetrazolium bromide (MTT) assay

{3-(Dimethylthiazol-2-yl)-2,5-Diphenyltetrazoliumbromide} (500 mg) was suspended in 50 ml of phosphate-buffered saline (PBS) to ready a 5 mg/ml concentration of the stain (as stock solution). To remove any blue formazan product, the solution was filtered through a 0.2 µm syringe filter before being kept in sterile, opaque, screw-capped vials in a deep freezer. Each microtiter plate well containing human lymphocytes that had been exposed to various concentrations of GT for 24 hours received ten µl of the MTT dye. The optical density of each well was measured using an ELISA reader at a transmitting wavelength of 620 nm by suspending the MTT-formazan crystals, which are exclusively formed by live cells, in 100 µl of dimethyl sulphoxide (DMSO) (19). The cytotoxic effects were measured based on Wang et al (20) as follows:

Inhibition of growth % = OD of control- OD of Sample /OD of control* 100

Preparation of human breast carcinoma (AMJ13) and brain cancer (AMGM)

Human brain cancer (AMGM) and breast cancer (AMJ13) were obtained from the Iraqi center for cancer and medical genetic researches, and washed in PBS, minced into small (2 mm) pieces, and digested with warm Trypsin. The undigested tissue was then removed from the cell suspension by filtering it through sterile mesh, and the filtrate was then centrifuged at 1,500 rpm for 10 minutes. The resulting pellet was suspended in RPMI-1640 medium that had 20% fetal bovine serum, 100 IU of penicillin, and 100 g of streptomycin added as supplements. After that, the suspension was placed in a falcon-shaped plastic container and incubated at 37°C. Every three to four days, the tumor cells were passaged, and a few passages were frozen in liquid nitrogen (21).

Measurement of cytotoxicity assay using Crystal violates stain:

To make a stock solution, 5 g of crystal violet was dissolved in 50 ml of formaldehyde 37% and 200 ml of methanol. Then, 10 ml from the stock solution was added to 90 ml of PBS (as 1 volume: 10 volume), and filtered through a filter used for cell culture staining and fixation. The medium was taken off the plate and given three warm PBS washes. At the conclusion of the final 20 minutes of incubation, the dye was withdrawn from the plate, and the wells were rinsed with tap water until the plate was clean. A 0.1ml of Crystal violate working solution dye was added to each well. Lastly, the plate was prepared for readout by an ELISA reader at 492 nm after drying (22).

Statistical analyses

The collected data was described and analyzed using the SPSS 26.0 program. The independent *t*-test was used to determine the differences between mean values. A probability of $p \leq 0.05$ and ≤ 0.01 were considered statistically significant and highly significant respectively (23).

Results

Collection and identification of *Candida* isolates

Throughout the six-month test period, 110 (23.15%) isolates of *Candida* species were collected and identified. The results show that there were 65 males (59.1%) and 45 females (40.9%), with an age range of 30 days – 78 years. For infants, children, and teenagers there were higher percentages among males than females, whereas the opposite was observed for the age groups between 21 and 60 years. Male preponderance was observed after 60 years of age. These results were statistically non-significant, Table (1).

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

Age (Years)	Gender		Total No (%)
	Males - No (%)	Females - No (%)	
< 1	11 (24.4)	4 (6.2)	15 (13.6)
1-10	4 (8.9)	4 (6.2)	8 (7.3)
11-20	4 (8.9)	3 (4.6)	7 (6.4)
21-30	6 (13.3)	11 (16.9)	17 (15.5)
31-40	5 (11.1)	15 (23.1)	20 (18.2)
41-50	7 (15.6)	13 (20.0)	20 (18.2)
51-60	5 (11.1)	5 (7.7)	10 (9.1)
>60	3 (6.7)	10 (15.4)	13 (11.8)
Total	45 (100)	65 (100)	110 (100)
Chi-Square Tests		*P*-value	0.094 N.S

The distribution of the types of *Candida* by the age groups of patients is shown in table (2). The table shows that the highest frequency of cases occurred in those 31-40 and 41-50 years with 20 cases each (18.2%). This is followed by those 21-30 years old (17, 15.5%), infants (15, 13.6%), in older adults>60 years (13, 11.8%), and others. The highest occurrence was that of *C. albicans* isolates (72, 65.5%), with all the non-*albicans* isolates being (38, 34.5%). The multiple cells in the table with low frequencies were merged to make the Chi Square test more feasible to use ($p < 0.001$).

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

Age group	No. and % of Candida Isolates		Total
	C. albicans	*Other Candida*	
< 1	7 (9.7)	8 (21.1)	15 (13.6)
1-10	3 (4.2)	5 (13.2)	8 (7.3)
11-20	4 (5.6)	3 (7.9)	7 (6.4)
21-30	17 (23.6)	0 (0.0)	17 (15.5)
31-40	19 (26.4)	1 (2.6)	20 (18.2)
41-50	11 (15.3)	9 (23.7)	20 (18.2)
51-60	6 (8.3)	4 (10.5)	10 (9.1)
>60	5 (6.9)	8 (21.1)	13 (11.8)
Total	72 (100)	38 (100)	110 (100)
Chi-Square Tests		P-value	0.001**

Detection and Characterization of Secondary metabolites GT using TLC and HPLC analysis

Our results of TLC indicate that the R_f 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).

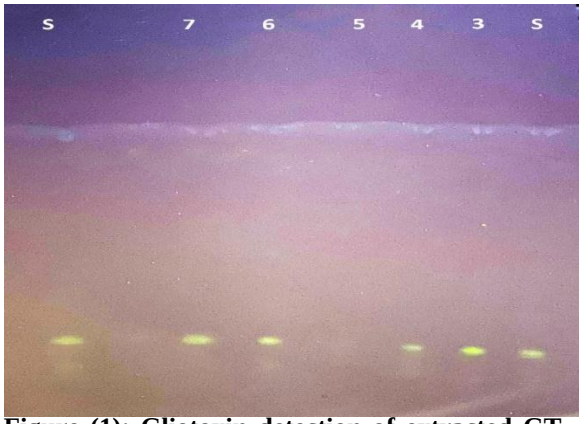
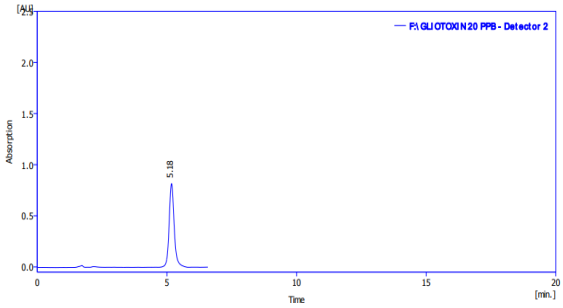


Figure (1): Gliotoxin detection of extracted GT from *C. albicans* isolates using a TLC plate (numbers 3-7) compared to standard GT (S)

A



B

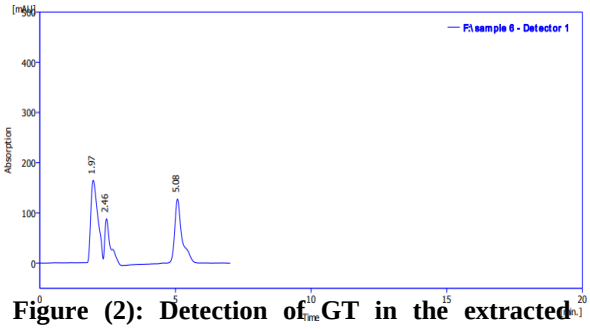


Figure (2): Detection of GT in the extracted culture supernatant, analysed using HPLC results. A-GT had a retention time of the main peak of 5.18 min as reported by injection of a GT standard. B- GT had a retention time of the main peak of 5.18 min as reported by injection of an extracted culture supernatant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

Cytotoxic effect of GT against lymphocytes and cancer cell lines

Cytotoxic effect of GT against lymphocytes

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.

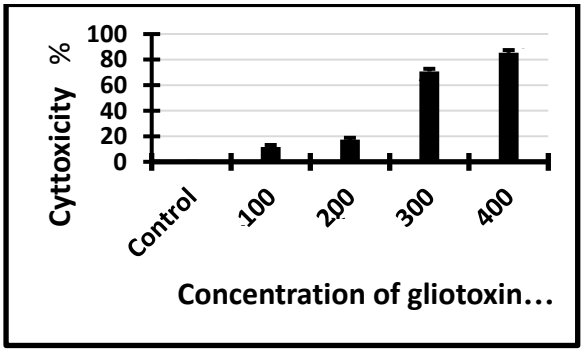


Figure (3): Cytotoxicity effects of different concentrations (100-400 µg/mL) of gliotoxin on lymphocytes for 24 hours on MTT test.

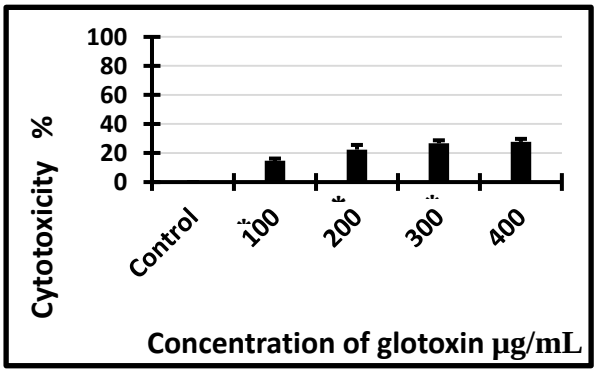


Figure (4): Cytotoxicity effect of different concentrations (100-400 µl/mL) of gliotoxin on AMGM cells for 48 hours by crystal violate test

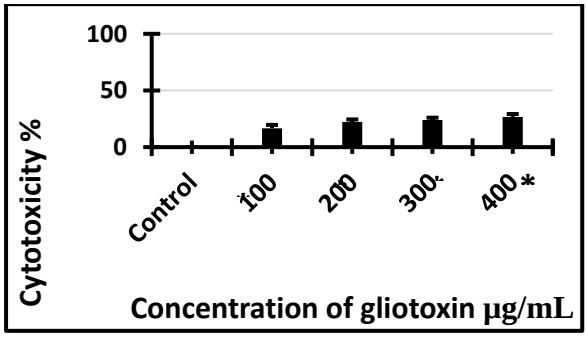


Figure (5): Cytotoxicity effect of different concentrations (100-400 µl/mL) of gliotoxin on AMJ13 cells for 48 hours by crystal violate test

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, pseudo-hyphae, 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 R_f value of extracted gliotoxin from investigated isolates was 0.38, in consistence to the results of Jayalakshm et al., 2021 (34), who reported that the R_f 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. tropicals*, 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. fumagutus*, 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 IC_{50} 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 IC_{50} 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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التأثير السام للخلايا الغليوتوكسن من صنف المبيِّضات المعزولة من مصادر سريرية ضد السرطان وخطوط الخلايا الطبيعية

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الخلاصة

خلفية البحث: أصبحت العدوى الفطرية الغازية أكثر شيوعًا خلال العقدين الماضيين. أنواع المبيضات، هي أكثر أنواع العدوى الفطرية البشرية شيوعًا. يتميز بإصابات داخلية بسبب عوامل الضراوة، مثل سم الجليوتوكسين وهو نوع من السموم الفطرية التي يعتقد أنها مضادة للبكتيريا والفطريات والفيروسات.

هدف الدراسة: كان الهدف من الدراسة الحالية هو اختبار قدرة أنواع المبيضات المعزولة من المصادر السريرية على إنتاج سم الجليوتوكسين كعامل ضراوة والتحقيق في تأثيرات السمية الخلوية ضد بعض سلالات الخلايا المختارة.

المواد والطرق: تم جمع 110 عزلة سريرية من أنواع المبيضات، وتحديدها وتشخيصها من خلال طرق المختبر الروتينية والاوساط الزرعية. تم اختبار وقياس قدرة عزلات المبيضات على إنتاج سم الجليوتوكسين بالطرق التحليلية. تم تطبيق السمية الخلوية للسم الجليوتوكسين الناتج ضد سلالات الخلايا الطبيعية والسرطانية.

النتائج: تم تحديد 110 عزلة على النحو التالي، المبيضات البيضاء، المبيضات الاستوائية، داء المبيضات *Candida parapsilosis*. 28 عزلة من المبيضات أظهرت إنتاج السموم الدبقية، وتم الإبلاغ عن تأثيرات السمية الخلوية للسم الجليوتوكسين الخلايا الليمفاوية وخطوط الخلايا AMGM و AMJ13 بتركيزات مختلفة. لوحظ أعلى تأثير سام للخلايا بتركيز 400 ميكروغرام / مل من السم الجليوتوكسين.

الاستنتاجات: أشارت النتائج إلى أن إمراضية الكانديدا كانت موزعة على جميع الأعمار من الجنسين وأنواع عديدة من العزلات الإكلينيكية. بعض العزلات كانت لديها القدرة على إنتاج سم الجليوتوكسين، كما أظهرت النتائج تأثير السم على الخلايا الطبيعية والسرطانية

الكلمات المفتاحية: المبيضات، سم الجليوتوكسين، HPLC، السمية الخلوية.