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Production and Identification of Secondary Metabolite Gliotoxin-Like Substance Using Clinical Isolates of *Candida* spp.

Noorulhuda Ojaimi Mahdi Al-Dahlaki and Safaa Al-Deen Ahmed Shanter Al-Qaysi*

Department of Biology, College of Science for (Women), University of Baghdad, Baghdad 10071, Iraq

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Most fungal infections by opportunistic yeast pathogens such as *Candida* spp. are the major causes of morbidity and mortality in patients with lowered immune. Previous studies have reported that some strains of *Candida* secret secondary metabolites play an important role in the decreasing of immunity in the infected patient. In this study, 110 *Candida* spp. were isolated from different clinical specimens from Baghdad hospitals. *Candida* isolates were identified by conventional methods, they were processed for *Candida* speciation on CHROMagar. The results of identification were confirmed by internal transcribed spacer (ITS) sequencing. Phylogenetic trees were analyzed with reference strains deposited in GenBank. Antifungal susceptibility testing was evaluated by the disc diffusion method and performed as recommended by the Clinical and Laboratory Standard Institute (CLSI) M44-A document. *Candida* isolates investigated produce secondary metabolites gliotoxin with HPLC technique and quantification. Out of 110 *Candida* isolates, *C. albicans* (66.36%) was the most frequent isolate, followed by the isolates of *C. tropicalis* (10.9%) and *C. glabrata* (6.36%) respectively. Concerning the antifungal susceptibility test, *Candida* isolates showed a high level of susceptibility to Miconazole (70.9%), Itraconazole (68.2%), and Nystatine (64.5%). The ability of obtained isolates of *Candida* spp. to produce gliotoxin on RPMI medium was investigated, only 28 isolates had the ability to secret this toxin in culture filtrates. The highest concentrations were detected in *C. albicans* (1.048 µg/ml). Gliotoxin productivity of other *Candida* species was significantly lower. The retention time for gliotoxin was approximately 5.08 min.

Keywords: ***Candida* species, gliotoxin, virulence factors, CHROMagar**

Introduction

Gliotoxin (GT) is a sulfur-containing mycotoxin, produced by various fungal species such as *Aspergillus fumigatus*, *Trichoderma virens*, *penicillium* spp., *Gliocadium fimbriatum,* and *Candida* spp. [1–4]. The fungal metabolite (GT) is an epipolythiodioxopiperazine (ETP) and is considered an antimicrobial, antifungal, and antiviral agent, this type of fungal toxin possesses a disulfide bridge across a piperazine ring that appears to be involved in the toxic effects. *Candida* species are yeasts that inhibit different sites as commensal microflora of humans and animals [5]. This yeast can invade and colonize the mucosal membrane of vaginal, oral surfaces and cavities, gastrointestinal tract and can be present on the skin. *Candida* spp. it rarely exists in the environment outside human tissues and the body. Several virulence factors have been attributed to the genus of *Candida* spp. which plays a role in the change from commensalism to disease, Candida infections are major causes of morbidity in compromised human hosts, but an understanding of the pathogenesis and virulence of this yeast remains incomplete [5, 6].

***Corresponding author**
Phone: +009647809749633
E-mail: Safaaa_bio@csw.uobaghdad.edu.iq

Infections by *Candida* have increased dramatically over the past decades in patients undergoing therapy of immunosuppressive antineoplastic or therapies associated with organ transplantation, patients suffering from cancer, and use several types of broad-spectrum antibiotics [7]. The possibility of production of mycotoxin metabolites (gliotoxin; GT), which are poorly studied and reported to possess immunomodulating, antiphagocytic, and antithrombotic properties, may be produced by this yeast investigated [8, 9]. The toxic effects of GT on the immune system have been reported in vitro, Mycotoxin GT plays an important role in the induction of apoptosis among thymocytes, peripheral lymphocytes, and cytoskeletal changes that affect macrophage properties and function [10–13].

Since then, many reports and studies have focused on the biological activities of GT in mammalian cells, through the formation of mixed disulfides with cell proteins and inhibiting the transcription factors of the nuclear factor kappa B (NF-κB) and the 20S proteasome activity, which might account for the immunosuppressive properties of this mycotoxin [12]. Also, GT has been shown to inhibit some enzymes such as creatine kinase of the rabbit muscle cell, alcohol dehydrogenase, and acetolactate synthase of higher plants [14, 15]. *In vitro* GT has been shown to prevent and inhibit cell growth or to display cytotoxicity with several types of cell lines [16–18]. Already in 1947, the anticancer activity of GT was demonstrated, and in 2004 it was found that GT was a very active inhibitor against six breast cancer cell lines with IC_{50} values between 38 and 985 nM [19]. Therefore, the production of secondary metabolites gliotoxin has been documented in various fungal genera as described above, but in yeasts very poor like *Candida* spp. isolated from clinical infections and commensal, this study aimed to investigate the ability of clinical isolates of *Candida* spp. to secret gliotoxin obtained from various clinical sources and specimens.

Materials and Methods

Organisms

This study was performed on 110 *Candida* spp. isolates were collected from several clinical sources including oral cavity, Vaginal swab, Bloodstream, Urine, Ear and skin swab, and peritoneal fluid of patients from September 2021 to March 2022, All isolates were used to assess the antifungal susceptibility test and ability to the production of secondary metabolites gliotoxin.

Identification of *Candida* spp.

Candida spp. yeasts were identified according to genus and species using phenotypic characteristics on Sabouraud 2% (w/v) glucose agar (Himedia, India), germ tube formation in fresh human serum, according to [20, 21]. Chlamydospore formation on specific medium rice-polysorbate 80 agar (Himedia). CHROMagar medium (Oxoid) was used to differentiate between *Candida* species, this medium was prepared according to the manufacturer's instruction and dispensed into sterilized pert dishes. The collected *Candida* isolates were inoculated in parallel onto CHROMagar, the culture plates were incubated at 37℃ for 24 h. All clinical yeast isolates observed on this medium were identified by morphological characteristics and pigmentation according to the manufactures instructions and as described by [22, 23].

Molecular identification

DNA extraction from *Candida* spp. For DNA extraction, the highest produced isolates were identified using Polymerase Chain Reaction (PCR), these isolates were cultured in 3 ml of YPD medium (2% peptone, 1% yeast extract, and 2% dextrose) and incubated at 30℃ for 24 h. *Candida* isolates were retrieved from the culture by centrifugation at 8600 ×*g* for 1 minute, the supernatant was discarded. Genomic DNA from *Candida* isolates was extracted by a yeast DNA Kit (Promega, USA), according to the manufacturer's instructions. The concentration and purity of extracted DNA were measured by Nanodrop. The reaction of PCR was prepared to a final volume of 50 μl, containing reaction buffer 2.2 mM of $MgCl_2$, 200 μM of each dNTP, 2.5 unit of Taq DNA polymerase, a 30 ng DNA template, and 50 pmol of each primer. An initial denaturation step for 5 min at 95℃ was followed by 30 cycles of denaturation at 94℃ for 40 sec. annealing at 58℃ for 40 sec and extension at 72℃ for 5 min. PCR product was applied on agarose gel electrophoresis. For PCR product sequencing universal forward and reverse primers of ITS1 5'-TCCGTAGGTGAACCTGCGG-3' and ITS4 TCCTCCGCTTATTGATATGC-3' were used to amplify the DNA fragment of the ITS1-5.8S-ITS2 region (Internal transcript space

region) as described by [24]. Bidirectional sequencing of PCR products was performed by (Macrogen Inc., Korea). The obtained sequences were blasted and aligned to compare with reference data in GeneBank. The sequences were submitted to GeneBank and their corresponding accession numbers were obtained. Phylogenetic trees were built using the neighbor-joining method in comparison of sequences of obtained isolates with the references strains was achieved using MEGA X software.

Antifungal susceptibility testing. Antifungal susceptibility of the *Candida* spp. isolates were determined using the Agar disc diffusion method according to Clinical and Laboratory Standards Institute (CLSI) [25]. Seven standard antifungal discs including Clotrimazole CC (10 µg/disc), Ketoconazole KT (10 µg/disc), Voriconazole VRC (10 µg/disc), Nystatin NS (50 µg/disc), Miconazole MIC, Amphotericin-B AP (20 µg/disc), Itraconazole IT (10 µg/disc) were purchased from (Bioanalytics, Turkey). A suspension of *Candida* spp. isolates were prepared by picking some colonies from the SDA culture plates from a 24 h old culture of yeast. Colonies were then inoculated in 5 ml of sterile normal saline, turbidity of all tubes was adjusted to 0.5 McFarland standard using Biomerieux DensiCHEK plus turbidity meter. A suspension of *Candida* spp. 10^7 cells/ml was spread onto Muller Hinton agar MHA surface supplemented with 2% glucose and 0.5 µg/ml methylene blue dye using cotton wool swabs. Antifungal discs were applied to the inoculated Petri dishes using sterile forceps. Then, the plates were incubated aerobically in an incubator at 37℃ for 24 h. The diameters of the halo zone were measured in millimeters. The results were an interpretation of the antifungal susceptibility was done according to CLSI standard guidelines for the detection of resistance, dose-dependent, and sensitive *Candida* strains.

Production of secondary metabolites GT using *Candida* spp. isolates. All *Candida* spp. isolates were grown on SDA medium at 37℃ in an incubator for 24 h. To evaluate the biosynthesis of GT in these yeast isolates, the inoculum was prepared by sampling 6 yeast colonies with a sterile loop, which was then transferred to 10 ml sterile normal saline. The inoculum was mixed using vortex for 5 minutes, and 1 ml was transferred to sterile Roux culture bottles (250 ml) with 100 ml of RPMI 1460 medium supplemented with 5% (V/V) fetal calf serum (Sigma, Germany), and then incubated at 37℃ for 7 days in a humified incubator with 5% CO_2. After that, the produced GT was extracted with 70 ml of chloroform. The mixture was centrifuged for 30 min at 5500 ×*g*, and the chloroform fraction was evaporated in an evaporator at 50℃. The dry extract was dissolved in 200 ml methanol. Then, filtrated through a Millipore filter unit (0.45 µm).

HPLC analysis and quantification of GT. High-performance liquid chromatography (HPLC model, SYKAM-Germany) was used for the detection and quantification of GT in culture extracts as described by [26]. A C-18 column (25 cm × 4.6 mm × 100 mm) particle size of 5 µm, (Kunaer, Germany) was used for setting the temperature at 30℃. HPLC analysis of extracted samples was prepared as gradient elution using H_2O-CH_3OH: 0–10 min, 55% H_2O; 10–11 min, 40% H_2O, 11–20 min, 40% H_2O, 20–22 min, 55% H_2O. Then, the flow rate was adjusted to 0.8 ml/minute and the injection volume was 20 µl. For detection, An UV signal detector (244 nm Detector, Waters) at a flow rate of 2.0 ml/min was used, Analyses and system control software (Claritychrom, V7.4.2.107) and monitoring the absorbance at 273 nm. A standard curve was obtained with a standard GT (Sigma-Aldrich, Germany) ranging from 62.5–8000 ng. Mean interassay and intra-assay coefficients of variations over the range of the standard curve were < 10%. The overall recovery of GT standard from culture supernatant was 70.5%, this was determined as the ratio of the defined amount of GT standard extracted from the RPMI culture medium with subsequent HPLC quantification and the same amount of GT standard directly injected into the HPLC system. The limit detection of the HPLC system was less than 10 ng/ml GT as assessed by serial dilution of GT standard in culture medium with subsequent GT extraction and quantification by HPLC.

Statistical analyses. The SPSS 22.0 software for Windows (SPSS Inc., USA) was used for statistical analyses. t-test analyses examine the difference between samples that were collected from males and females as well as the isolation sources, differences were considered

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Results

Isolation and identification of *Candida* spp.

During the 6-month study period, 475 non-duplicated clinical samples of suspected patients with candidiasis were collected from several hospitals in the capital of Baghdad, Iraq. A total of 110 (23.15%) isolates of *Candida* were recovered from the collected clinical samples. The *Candida* isolates distribution was as follow according to the site of isolation, sputum, (*no* = 52, 47.32%), urine, (*no* = 33, 29.7%) and vaginal, (*no* = 16, 14.4%), Folly for urethra (*no* = 2, 18.2), Blood (*no* = 2, 1.82%), Peritoneal fluid (*no* = 1, 91%), Left renal aspirate fluid (*no* = 1, 0.91%), and Mouth cavity (*no* = 3, 2.72%) respectively. Distribution of *Candida* spp. isolates between the two genders showed that (*no* = 61, 55.45%) was isolated from females and (*no* = 49, 44.54%) from males. The frequency distribution of the collected isolates according to gender shows significant differences ($p < 0.01$) as shown

Table 1. Distribution frequency of *Candida* species isolated from various clinical specimens according to the source of isolation and gender.

Type of sample	No. (%)	Female No. (%)	Male No. (%)	*P*-value
Sputum	52(47.32)	24(21.6)	28(25.2)	0.05
Urine	33(29.7)	19(17.1)	14(12.6)	0.05
Vaginal	16(14.4)	16(14.4)	-	0.065
Folly for urethra	2(1.82)	-	2(1.82)	0.073
Blood	2(1.82)	1(0.91)	1(0.91)	0.05
Peritoneal fluid	1(0.91)	-	1(0.91)	0.05
Left renal aspirate fluid	1(0.91)	-	1(0.91)	0.086
Mouth cavity	3(2.72)	1(0.91)	2(1.82)	0.05
Total	110(100)	61(55.45)	49(44.54)	< 0.05*

Data are presented as number (n) with proportion (%) unless indicated otherwise.

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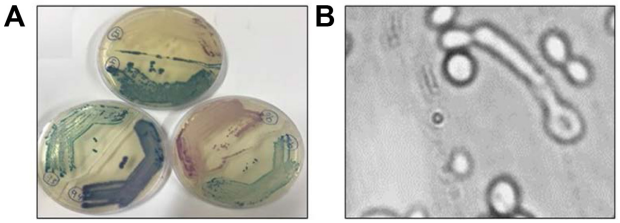

Fig. 1. (A) Appearance of *Candida* spp. colonies on CHROMagar. (B) Formation of *C. albicans* Germ tube in fresh human serum incubated for 3 hours to form a germ tube at 100X magnification.

in (Table 1).

Results of primary identification of *Candida* spp. based on the morphological characteristics, growth of this yeast on CHROMagar medium, germ tube formation, and chlamydospore development, revealed that nine types of *Candida* spp. were identified (Fig. 1). The frequency distribution of the *Candida* isolates according to species shows highly significant differences ($p < 0.001$), *C. albicans* was the most predominant encountered species (*no* = 73, 66.36%), and the most predominant isolates were collected from sputum (*no* = 52, 47.32%) and urine (*no* = 33, 29.7%), respectively. While, only one isolate of *C. albicans* was collected from Folly for urethra Left, peritoneal fluid, and renal aspirate fluid. The predominance of non-*albicans Candida* species (*no* = 37, 33.63%) was collected among the positive samples, these isolates were relatively smaller than *C. albicans* detected in the present study. Seven different types of non-*C. albicans* species were collected, which were *C. tropicalis* (*no* = 12, 10.9%), *C. parapsilosis* (*no* = 4, 3.6%), *C. kyfer* (*no* = 6, 5.5%), *C. krusei* (*no* = 5, 4.5%), *C. glabrata* (*no* = 7, 6.36%), *C. rugosa* (*no* = 2, 181%), and *C. lusitaniae* (*no* = 1, 0.9%). In this study, a high rate of non- *C. albica* was found in urine and sputum, while the low positive rate was obtained from virginal and Mouth cavity. The number of different *C. albicans* and non-*C. albicans* species concerning the source of isolation in our study are presented in (Table 2).

For more conformation, all isolates were subjected to identification using the VITEK2 system and molecular techniques, molecular identification, and the phylogenetic analysis of *Candida* spp. isolates were achieved using ITS1-ITS4 sequencing for fungal identification was performed. The highest producer isolates of GT include *C. albicans, C. tropicalis, C. parapsilosis,* and *C. glabrata* with accession numbers (ON853559), (ON853560), (ON853558), and (ON853558), respectively, were submitted to National Center for Biotechnology Information (NCBI). *C. albicanthe s* isolate showed 99.8% similarity with reference strain under accession number KY101886. While *C. tropicalis* showed 99.8% similarity with reference strain deposited in GenBank under accession number KX664646. The isolate of *C. glabrata* showed 100% similarity with the reference strain under accession number MF187319. An Isolate of *C. parapsilosis* revealed 99.8% similarity with strain under accession number LC390056. Phylogenetic trees of the four isolated *Candida* spp. isolates *C. albicans, C. tropicalis, C. glabrata* and *C. parapsilosis* with reference strains in GenBank can be seen in (Figs. 2A–D respectively).

Table 2. Distribution of *Candida* Isolates collected from various clinical specimens.

Clinical specimens	*Candida* spp.								Total	*p*-value
	C. albicans	*C. tropicalis*	*C. parapsilosis*	*C. kyfer*	*C. krusei*	*C. glabrata*	*C. rugosa*	*C. lusitaniae*		
Sputum	38	4	1	3	2	3	1	-	52	0.996
Urine	17	7	1	3	3	2	-	-	33	0.960
Vaginal	13	1	-	-	-	2	-	-	16	0.999
Folly for urethra	1	-	-	-	-	-	-	1	2	1.000
Blood	-	-	2	-	-	-	-	-	2	0.495
Peritoneal fluid	1	-	-	-	-	-	-	-	1	1.000
Left renal aspirate fluid	1	-	-	-	-	-	-	-	1	0.835
Mouth cavity	2	-	-	-	-	-	1	-	3	0.618
Total No. (%)	73(66.36)	12(10.9)	4(3.6)	6(5.5)	5(4.5)	7(6.36)	2(1.81)	1(0.9)	110	***$p < .001$

Antifungal susceptibility testing

Overall, the antifungal diffusion method was used to determine the resistance of *Candida* spp. isolated in the current study to common antifungal drugs. Antifungal susceptibility test of *Candida* spp. against Clotrimazole was found to be 55.5% susceptible (S), 10% susceptible dose-dependent (SDD), and 34.55% resistant (R). The results of antifungal susceptibility revealed that *Candida* spp. was highly susceptible to Itraconazol and Miconazole, 68.2% and 70.9% respectively, as shown in (Table 3). While the highest level of resistance among *Candida* spp. was reported to Voriconazole and Amphotericin-B, 50% and 45.4% respectively. For *C. albicans* the highest susceptibility was reported for Miconazole (*no* = 55, 75.3%), Itraconazol (*no* = 53, 72.6%), Nystatin (*no* = 47, 64.4%). Among the azole class, Voriconazole showed the

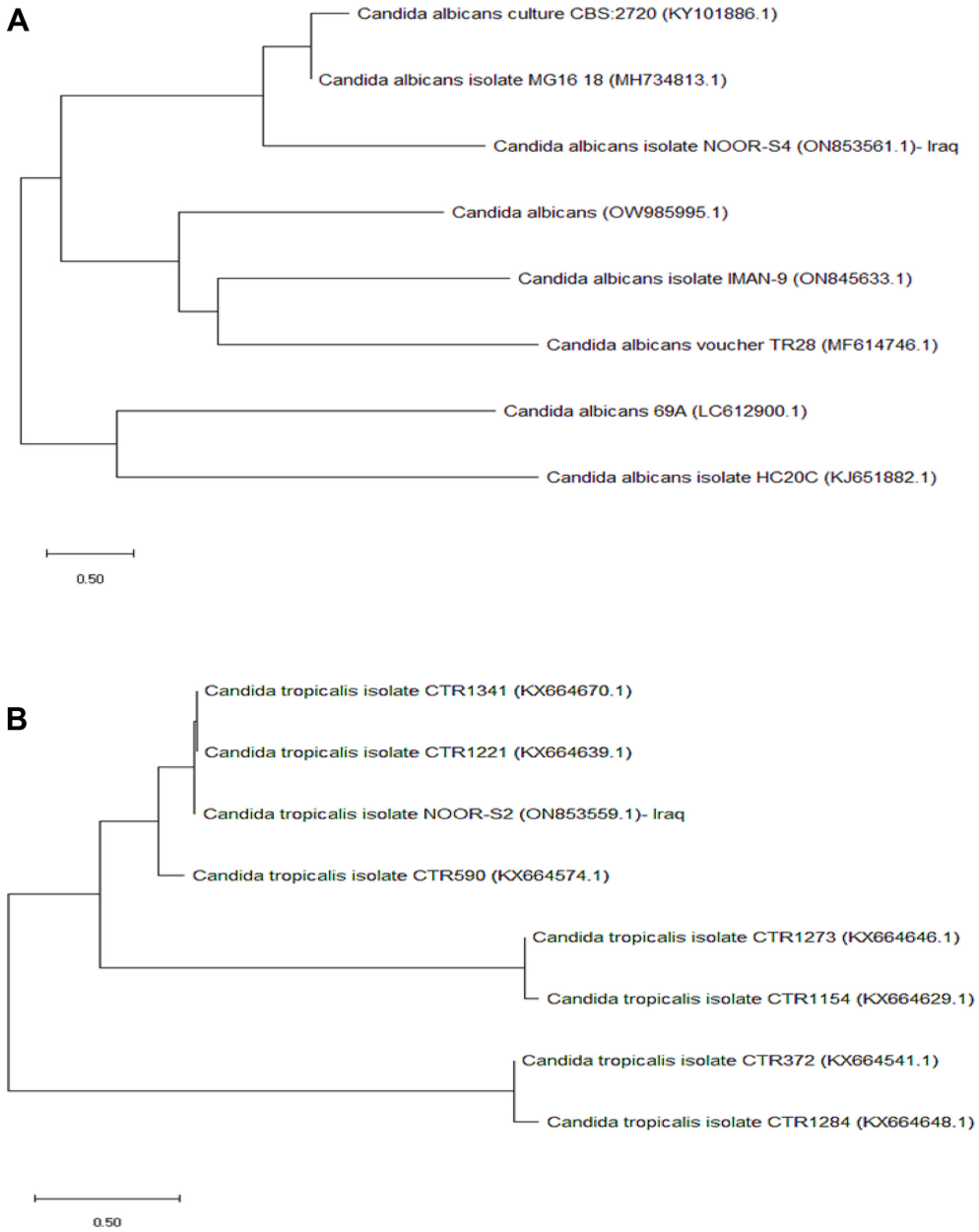

Fig. 2. Phylogenetic tree of (A) *C. albicans,* (B) *C. tropicalis* (C) *C. glabrata,* and (D) *C. parapsilosis* based on the 18S rRNA gene, ITS1, 5.8S rRNA, ITS2, and 28S rRNA sequences showing the relationship of *Candida* spp. isolates to closet other reference strains in Gene Bank, using the neighbor-joining method with software MEGA-X.

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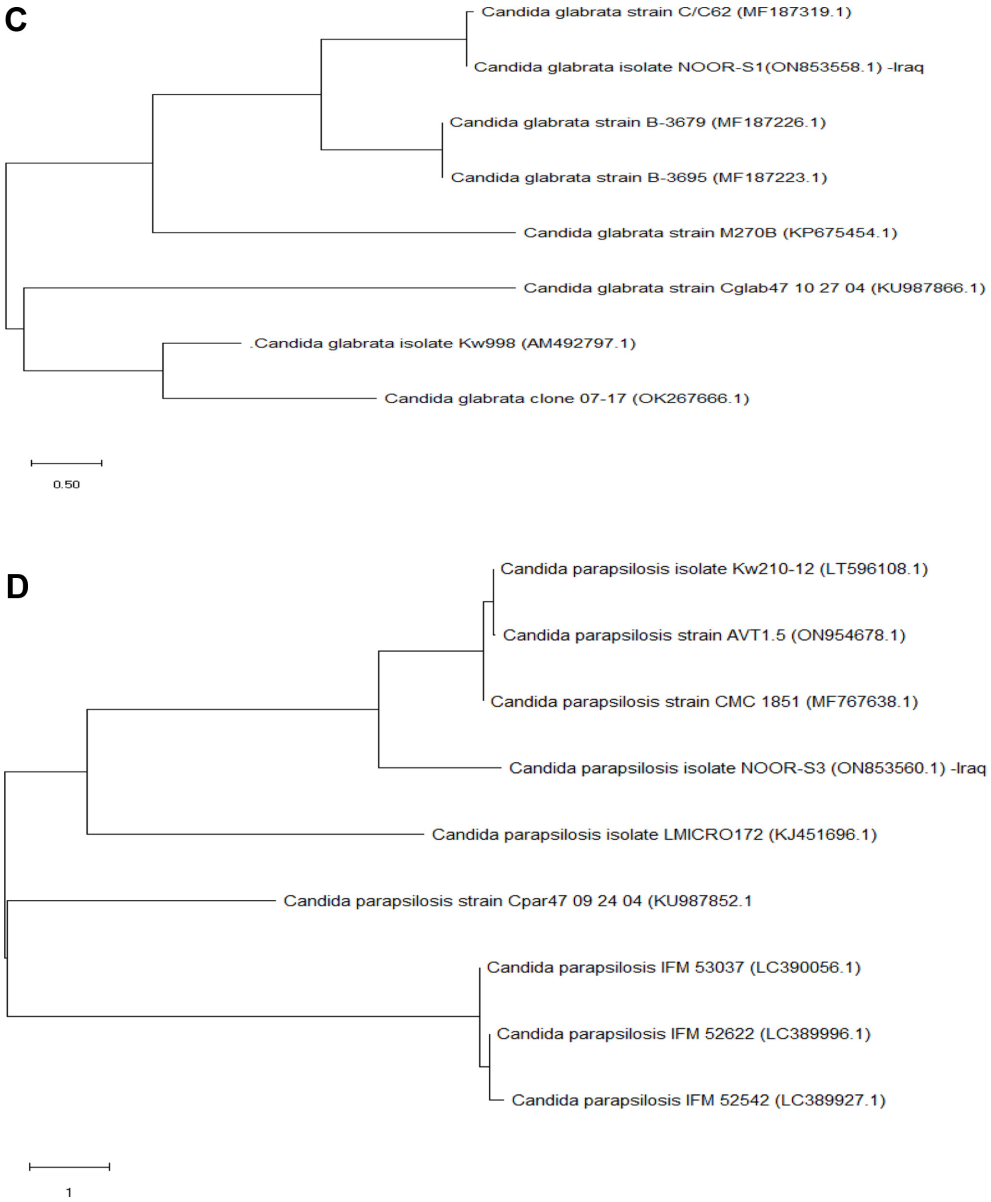

Fig. 2. Continued.

lowest susceptibility with 43.83% out of 73 *C. albicans* tested isolates, while 6.85% of isolates were susceptible dose-dependent, and 49.32% resistant. Also, *C. rugosa* and *C. lusitaniae* (100%) was found to be more susceptible to four antifungal which includes Clotrimazole, Itraconazol, Miconazole, and Ketoconazole, respectively. *C. albicans* isolates were found to be more susceptible to Miconazole, 75.2%, and Itraconazol, 72.6%. *C. tropicalis* and *C. parapsilosis* were found to be more susceptible to Ketoconazole, 75%. Whereas any resistance was reported among isolates of *C. rugosa,* and *C. lusitaniae* to each of Clotrimazole, Nystatin, Itraconazol, Ketoconazole, and Miconazole respectively. The antifungal susceptibility profile of all tested antifungal drugs against the *C. albicans* and non-*C. albicans* is summarized in (Table 3).

Production of secondary metabolites GT using *Candida* spp. isolates

In a preliminary investigation, the ability of all *Candida* isolates including *C. albicans, C. tropicalis, C.*

Table 3. Antifungal susceptibility testing of all *Candida* isolates against some antifungal agents according to CLSI.

Clinical specimens		*Candida* spp. Total (Number = 110 isolate)								Total No (%)
		C. albicans No = 73	*C. tropicalis* No = 12	*C. parapsilosis* No = 4	*C. kyfer* No = 6	*C. krusei* No = 5	*C. glabrata* No = 7	*C. rugosa* No = 2	*C. lusitaniae* No = 1	
Clotrimazole	S*	43(58.9%)	5(41.7%)	2(50%)	2(33.3)	3(60%)	3(42.85%)	2(100%)	1(100%)	61(55.5%)
	SDD*	6(8.3%)	4(33.3%)	0	0	0	1(14.3%)	0	0	11(10%)
	R*	24(32.8%)	3(25%)	2(50%)	4(66.7%)	2(40%)	3(42.85%)	0	0	38(34.55%)
Voriconazole	S	32(43.83%)	5(41.7%)	1(25%)	2(33.3%)	3(60%)	3(42.85%)	1(50%)	0	47(42.7%)
	SDD	5 (6.85%)	1(8.3%)	0	1(16.7)	1(20%)	0	0	0	8(7.3%)
	R	36(49.32%)	6(50%)	3(75%)	3(50%)	1(20%)	4(57.15%)	1(50%)	1(100%)	55(50%)
Nystatine	S	47(64.4%)	8(66.6%)	3(75%)	5(75%)	1(20%)	5(71.4%)	1(50%)	1(100%)	71(64.5%)
	SDD	6(8.3%)	2(16.7%)	0	1(25%)	0	0	0	0	9(8.2%)
	R	20(27.3%)	2(16.7%)	1(25%)	0	4(80%)	2(28.6%)	1(50%)	0	30(27.3%)
Itraconazol	S	53(72.6%)	7(58.3%)	3(75%)	3(50%)	3(60%)	3(42.8%)	2(100%)	1(100%)	75(68.2%)
	SDD	6(8.3%)	2(16.7%)	0	2(33.3%)	0	2(28.6%)	0	0	12(10.9%)
	R	14(19.1%)	3(25%)	1(25%)	1(16.7)	2(40%)	2(28.6%)	0	0	23(20.9%)
Ketoconazole	S	40(54.8%)	9(75%)	3(75%)	2(33.3%)	3(60%)	2(28.6%)	2(100%)	1(100%)	62(56.4%)
	SDD	14(19.1%)	2(16.7%)	0	0	0	0	0	0	16(14.5%)
	R	19(26.1%)	1(8.3%)	1(25%)	4(66.7%)	2(40%)	5(71.4%)	0	0	32(29.1%)
Miconazole	S	55(75.3%)	7(58.3%)	2(50%)	2(33.3%)	5(100%)	4(57.1%)	2(100%)	1(100%)	78(70.9%)
	SDD	9(12.35%)	3(25%)	0	1(16.7)	0	2(28.6%)	0	0	15((13.6%)
	R	9(12.35%)	2(16.7%)	2(50%)	3(75%)	0	1(14.3%)	0	0	17(15.5%)
Amphotericin-B	S	16(21.9%)	4(33.33%)	3(75%)	0	3(60%)	2(28.6%)	0	0	28(25.5%)
	SDD	20(27.4%)	1(8.33%)	0	3(50%)	2(40%)	4(57.1%)	1(50%)	1(100%)	32(29.1%)
	R	37(50.7%)	7(58.33%)	1(25%)	3(50%)	0	1(14.3%)	1(50%)	0	50(45.4%)

*S, Susceptible; SDD, Susceptible dose dependent; R, Resistance.

parapsilosis, C. kyfer, C. krusei, C. glabrata, C. rugosa, and *C. lusitaniae* to produce GT was investigated after 7 days of cultivation in RPMI-1640 medium supplemented with 5% fetal calf serum at 37℃. HPLC was performed to determine the production of GT in the culture supernatant of *Candida* isolates. The results of HPLC analysis revealed that the GT standard had a retention time of 5.18 min, the standard calibration curve was determined by various 2-fold diluted concentrations of GT standard. Chromatographic peaks of GT in the culture supernatants of *Candida* isolates were detected in 28 isolates by HPLC, these isolates produced a peak with a chromatographic retention time consistent (narrow) with the GT standard 5.08 min (Supplementary Fig. S1). The results of HPLC chromatogram analysis show the highest producer isolates of *Candida* including *C. albicans, C. tropicalis, C. glabrata,* and *C. parapsilosis* respectively, which provided a chromatographic peak with a retention time identical to that of the GT standard. The amount of GT in the culture supernatants was calculated and quantified using an external standard calibration curve method, the results revealed that the highest concentration was 1.048 μg/ml produced by *C. albicans*, while the lowest concentration was 0.087 μg/ml produced by *C. prapsilosis*.

Discussion

The *Candida* species infections have become most common in hospitalized patients and their emergence is favored by immunosuppression. *Candida* spp. capable of causing multiple superficial and deep infections, particularly of the mucous membrane, skin, urinary and gastrointestinal tracts. Although *Candida* spp. are generally not very aggressive, they may cause infection if the immune system is weakened or if an environmental

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conditions become available. In this perspective, we focused part of our research on the epidemiology features of *Candida* spp. The collected *Candida* spp. isolates were identified and characterized by employing several methods. The results revealed that the most frequent species in the collected samples were *C. albicans* isolated over 6 months of period from different clinical specimens. The majority of *Candida* spp. were obtained from sputum and urine, which represents about 77%, which indicates the higher prevalence and distribution of *Candida* spp. causing respiratory tract and urinary tract infections due to its pathogenic mechanism factors. The high rates of infection by *C. albicans* are due to its ability to grow in different morphological forms such as pseudo-hyphae, true hyphae, and unicellular budding yeast, which increase its virulence and invading host cell activity [27, 28]. This result is consistence with the results of [29, 30], who reported that about 90% of *Candida* isolates were obtained from urine and sputum. *C. albicans* was the most common species among all *Candida* isolates is over 66% followed by *C. tropicals* 10.9%, *C. glabrata* 6.3%, and *C. kyfer* 5.5%. The obtained results show a high similarity to those previous results achieved by [29, 31, 32]. It is also documented by several researchers that the most predominant encountered species in *Candida* infections is *C. albicans* followed by *C. parapsilosis* [33, 34]. [35] Has revealed that the distribution of Candida spp. differs due to various environmental factors and the status of population's community.

The use of broad-spectrum antifungal and corticosteroids and the spread of immunodeficiency diseases were all associated with a dramatic increase incidence of harmful fungal diseases. Most fungal infections are treated by different classes of antifungals that include polyenes, Azoles, and echinocandins. All antifungal drugs target different biochemical pathways that are unique to yeasts. In the current study, *Candida* spp. isolates were found to be susceptible to Miconazole 70.9%, Itraconazol 68.2%, Nystatine 64.5%, Ketoconazole 56.4%, and Clotrimazole 55.5% respectively. Azoles are the most common antifungal drug classes used to treat and prevent *Candida* infections [36]. This class of antifungal target and interfering with the enzyme lanosterol 14α-demethylase (Erg11p) is an important enzyme in ergosterol biosynthesis which is a key component in the fungal cell wall [36]. The rates of resistance to azole e group have been reported via both acquired resistance and intrinsic resistance [37]. Polyenes class of antifungal drugs targets ergosterol in the plasma membrane and are fungicides. They bind to the plasma membrane through ergosterol and form pores. Pore formation causes rapid leakage of monovalent ions (Na^+, K^+, H^+ and Cl^-) and causes fungal cell death[38]. Acquired resistance to amphotericin-B is rare and resistance usually develops by selecting inherently less susceptible strains of fungi [37]. We reported that *Candida* isolates were resistant to Voriconazole 55% and Amphotericin-B 50% respectively. Variation of these resistance rates may result from differences in the patient population, prior exposure to azoles, and different breakpoint values. Recently, resistance to common antifungals has been reported in various *Candida* strains isolated from various clinical sources [35]. In addition, fungal strains isolated from immunocompromised patients have high resistance levels to antifungals because of use as prophylaxis [35, 36]. A similar report was conducted on the antifungal test of Miconazole and Clotrimazole as a drug of choice against fungal pathogens[39]. The obtained results were in agreement with another funding has reported that *Candida* spp. was more susceptible to Clotrimazole 82% followed by Fluconazole 64% and Miconazole 44% respectively [29]. Hence, based on our findings, we suggest azole class and nystatin, a good antifungal agent for treating *Candida* infections. Moreover, it is suggested the general people not take antifungal drugs without a suitable diagnosis of fungal infections using moderate techniques like molecular or MALDI TOF MS to correctly diagnose of *Candida* spp.

The pathogenicity of *Candida* infections has been studied extensively, in the past decades, among *Candida* spp. polymorphic *C. albicans* generally represent the most common species causing fungal infection in human and animal tissues such as oral candidiasis, vaginitis, systemic infections, candidemia, and cutaneous candidiasis. Nowadays a range of other species including *C. parapsilosis, C. glabrata, C. tropicals, C. kefyr, C. krusei, C. famata, C. guilliermondii,* and *C. dubliniensis* has emerged as important opportunistically infections fungi [40–42]. Gliotoxin is an antifungal mycotoxin produced by several pathogenic fungi such as *Aspergillus*, *Trichoderma, Gliocladium, Penicillium*, and *Candida* [4, 43].

In the field of GT production and pathogenicity, several studies previously suggested and reported that *Candida* spp. has the ability to produce immunosuppressive secondary metabolites gliotoxin [16]. GT has been reported to have immunosuppressive properties mainly directed at macrophage and neutrophilic white blood cells of human and laboratory animals. In the report by [44] who suggested the possibility that gliotoxin may play a significant role in the virulence and pathogenicity of *C. albicans*, this was clear when investigate three symptomatic vaginal Candidiasis samples from women who are colonized by *C. albicans* and discovered the quantities of bioactive GT in the samples of secretion were greater than 1 μg/g.

Overall, HPLC analysis confirmed that among a total of 110 clinical *Candida* isolates were grown on RPMI medium under standard conditions, only 28 isolates detected to produce extracellular GT. Our results revealed that there are a significant values of GT were produced in a liquid culture medium of clinical *Candida* spp. particularly *C. albicans*, *C. tropicals,* and *C. glabrata* isolates extracted with chloroform. Our funding is in agreement with the study of Shah and Larsen [4], who analyzed 50 strains of *Candida* obtained from clinical infections at different body sites, Only 32 were secret the potent immunosuppressive mycotoxin gliotoxin-like material after 7 days in a synthetic medium supplemented with 5% fetal calf serum. Gliotoxin was detected in clinical isolates of *C. albicans* isolates from women with vulvovaginitis. A study achieved by [45], which looked at *C. albicans* isolates obtained from the oral cavity of patients with oral thrush, revealed that all tested isolates of *C. albicans* were produced to GT at concentrations ranging between 0.02 and 1.5 μg/ml. But is contradictory to the study achieved by [26, 46], which reported that there is no mycotoxin gliotoxin produced in culture medium using various clinically and commensal isolates of *C. albicans, C. tropical, C. glabrata, C. kefyr,* etc. The correlation between mycotoxin gliotoxin production and virulence in *Candida* spp. was further strengthened by observation of the occurrence of gliotoxin in secretion collected from women infected with *C. albicans*. Some reports and studies have highlighted the role of secondary metabolites gliotoxin substancestance during hyphal growth, in the induction of apoptosis during infections with *Aspergillus* and *Candida* [47], and it causes apoptosis in immune cells including macrophages, neutrophils, thrombocytes, and eosinophils [11, 48].

In our study, we found that *C. albicans* was the more frequent species responsible for different Candidal infections followed by *C. tropicalis, C. glabrata,* and *C. kyfer*, while the *C. lusitaniae* was the lowest frequencies. Commonly used antifungal agents Miconazole, Itraconazol, Nystatine and Ketoconazole demonstrated a high rate of sensitivity, while Amphotericin-B was the least effective for *Candida* isolates. Also, According to the analytical methods used and results documented in this study, it could be concluded that the success of some isolates of *Candida* spp. obtained from clinical sources to secret Gliotoxtin in liquid medium RPMI-1640, these isolates include *C. albicans, C. tropicalis, C. glabrata,* and *C. parapsilosis*.

Author Contribution

The authors have made a substantial, direct, and intellectual contribution to the work and approve it for publication.

Conflict of Interest

The authors have no financial conflicts of interest to declare.

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