Activity Assessment of Tunisian Olive Leaf Extracts Against Survival and Virulence *in vitro* of *Candida glabrata*

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Abstract

C. glabrata has become a prominent pathogen less susceptible to azole antifungal agents when compared with other *Candida* spp.

Aims: Our work aimed to study some virulence factors of *C. glabrata* isolates and to test the activity of *Chemlali* Olive Leaf Extract (OLE) against virulence and the survival of clinical *C. glabrata* isolates.

Methods: The virulence factors of a collection culture of *C. glabrata* isolated from blood culture (n = 35), vulvovaginal (n = 21), and buccal site (n = 5) were analyzed in specific medium with and without OLE.

Results: All *C. glabrata* isolates tested were able to produce haemolysin and to form biofilm (n = 61), 98.3% of isolates were phospholipase producer and 96.7% were protease producer. No significant difference between Pz values was obtained from oral cavity, vulvovaginal and blood culture isolates (*p* value ≥ 0.05). *C. glabrata* growth was dramatically decreased after treatment with olive leaves extract and exhibited MIC₉₀ value in the range of 2-8 mg/ ml and MFC value range from 16 to 32 mg/ ml. There was a significant (*p* < 0.05) decrease observed in Pz value in the test strains of *C. glabrata* treated with 0.25xMICs of olives leaves compared to untreated control at equal cell density. OLE was able to reduce *in vitro* proteinase production by an average of 55.87%, by 46.85% for phospholipase activity, by 25.37% for haemolysin activity and only 2.43% to 39.13% biofilm formation was recorded.

Conclusion: *C. glabrata* isolates from Sfax University Hospital presented a high virulent power and these activities do not depend on the sampling sites but on possible evolutionary changes that may occur between different regions of the world. Olive leaves extract evaluated here is a potential therapeutic drug for the treatment of *C. glabrata* infection not only in the growth but also in inhibition of virulence factors.

Keys words: *Candida glabrata*; Virulence factors; Olive leaves extract; Antifungal drugs

Introduction

C. glabrata has become a prominent pathogen causing invasive candidiasis infections at our institution. It was the third most common member of the genus isolated [1] and is of

interest because it is less susceptible to azole antifungal agents, especially to fluconazole, when compared with other *Candida* spp. The therapy of this type of fungemia has been undetermined by resistance, variable efficacy between strains or species, toxicity, and requirement for long courses of treatment. A need for identifying alternative natural and safe sources of molecules, especially of plant origin, to treat these diseases has notably increased in recent years [2]. Recently, several studies focused on high-added-value compound of the Olive Leaves Extract (OLE) [3-5]. The Olive plant leaves are used in folk medicine as a remedy for eye infections, sore throat, urinary tract infections, kidney problems and backaches or headaches. It is also used as a hypotensive, emollient, febrifuge and styptic [6]. The leaves of the tree were reported to be potent for the treatment of malaria in 1854 [7]. Olive oil occupies also an important place in the Mediterranean diet due to its nutritional and dietetic qualities and the *Chemlali* variety from Sfax region had excellent nutritional characteristics in terms of sterol, aliphatic alcohol, and tocopherol content [8]. In the present study, we investigate the activity of *Chemlali* olive leaf cultivar against virulence and the survival of clinical *C. glabrata* isolates from blood culture, vulvovaginal, and buccal site.

Materials and Methods

Patients and isolates

Sixty one *C. glabrata* clinical isolates were collected from 46 patients from different anatomical sites. Thirty five strains were isolated from Blood Culture (BC) and five isolates from oral site. Eleven *C. glabrata* strains isolated from three or more consecutive episodes of *Candida glabrata* vaginitis obtained from patients with RVVC (Recurrent vulvovaginal Candidiasis). Additionally, 10 patients who had experienced a single episode of VVC (Vulvovaginal Candidiasis) in a 1 year period were analyzed; two reference strain *C. glabrata* ATCC90020 and CBS138 and one *C. albicans* reference strains ATCC90028 were included as controls in the present investigation. All the blood specimens from the patients were inoculated into Bactec PED bottles (Becton-Dickinson, USA), which were incubated in the Bactec^R

9050 automated culturing system (BD diagnostic Systems 9050, Oxford, UK) at 37°C for 15 days or until the bottles were positive by the colorimetric detection of CO₂ and vaginal and oral sampling were carried out through sterile swabs. Yeasts grown on SDA plates (Sabouraud Dextrose Agar) at 37°C for *Candida* species were identified according to their morphological characteristics and biochemical profiles. To identify the isolates they were inoculated onto Candiselect ID medium (Bio-Rad, France) and incubated at 37°C for 48 h. *C. glabrata* was initially identified by the green color of its colonies, which was subsequently confirmed by ID32C (Biomérieux, France) assimilation test and GLABRATA RTT (Fumouze Diagnostic, Paris France) and the susceptibility to fluconazole was determined by measuring the Minimum Inhibitory Concentration (MIC) obtained through the use of the E-test (Biomérieux, France).

Preparation of Olive Leaves Extracts (OLE)

Plant material of *Chemlali* variety collected from Sfax region, Tunisia, was used as sample for our investigation. Leaves (1g) were homogenized and extracted over night at 32°C with 20 ml of methanol (ProLABO, Paris) in a capped glass tube on an agitating plate at a constant stirring rate (280 rpm).

The crude extracts were concentrated in a rotary evaporator and then transferred into sterile vials with 10 ml of DMSO (1%) and finally were kept under refrigerated conditions until further use [9,10].

Detection of virulence factors in isolates of *Candida glabrata*

Proteinase assay: Production of extracellular proteinase was assessed by the method of Aoki, et al. [11] using BSA agar plates. Briefly, 60 mL of a solution containing MgSO₄·7H₂O 0.04 g, K₂HPO₄ 0.5 g, NaCl 0.2 g, yeast extract 0.2 g, glucose 4.0 g and Bovine Serum Albumin (BSA) (Sigma, USA) 0.5 g; was prepared and the pH was adjusted to 3.5 with 1 N of HCL. This solution was filter sterilized and mixed with 140 ml of autoclaved melted agar to prepare the BSA agar plates.

Amounts of 10 µL of yeast suspension 1.10⁶ cfu.mL⁻¹ (Colony Forming Unit) were spot inoculated on the BSA agar plates and incubated at 37°C for 5 days. After incubation, the plates were stained with 0.1% amido black dye and destained with 15% acetic acid, and the clear zone was measured.

Pz values were calculated in terms of the ratio of the diameter of the colony to the total diameter of colony plus zone of solubilization as described by Price, et al. [12]. The enzymatic activity was scored into four categories, a Pz of 1.0 indicated no enzymatic activity; a Pz between 0.99 and 0.90 indicated low enzymatic activity; Pz between 0.89 and 0.70 corresponded to moderate activity; and low Pz values < 0.69 meant high enzymatic activities. The *Candida albicans* ATCC 90028 strains was used as a positive control.

Hemolysis assay: Haemolysin production was evaluated using a modified method of Luo, et al. [13]. The blood agar plates were prepared by adding 7 ml of fresh sheep blood suspended

in sterile Phosphate Buffer Saline (PBS) to 100 mL of SDA supplemented with 3% (w/ v) glucose. Amounts of 10 µl of yeast suspension 1.10⁶ cfu.mL⁻¹ were spot inoculated on the blood agar plates and incubated at 37°C for 48 h. After incubation, plates were scored for the presence of translucent ring and or a greenish black halo circumscribing the inoculum growth. Haemolysin activity (Pz values) was calculated in terms of the ratio of the diameter of the colony to the total diameter of colony plus zone of halo using the method described by Price, et al. [12].

Phospholipase production: Phospholipase activity of *Candida* species were detected by egg yolk agar plate method [12]. The egg yolk medium consisted of 13.0 g SDA (Scharlau, Spain), 11.7 g NaCl, 0.11 g CaCl₂ and 10% sterile egg yolk. A loopful of an overnight yeast culture approximately 1.10⁶ cfu.mL⁻¹ determined through densitometer was aseptically inoculated onto the medium and incubated at 37°C for 5 days. Phospholipase activity was calculated in terms of the ratio of the diameter of the colony to the total diameter of colony plus zone of halo using the method described by Price, et al. [12]. The *Candida glabrata* ATCC CBS138 strain was used as a positive control.

Biofilm formation in vitro and biomass quantification: For each experiment, strains were subcultured on Sabouraud Dextrose Agar (SDA) for 48 h in 37°C. Cells were then suspended in YPG medium (1%Yeast, 1%Peptone, 2%Glucose) and the cellular density was adjusted to 1.10⁶ cfu.mL⁻¹ using a densitometer.

Standardized cell suspensions (200 µl containing 1.10⁶ cfu.mL⁻¹ in SDA) were placed into selected wells of 96-well polystyrene microtiter plates (Kartell. SPA, Italy) and incubated in 37°C on a shaker at 120 rpm/ min. At 24h, 50µl of fresh YPG medium was added. The preparations were then incubated for a further 24h. After this step, the medium was aspirated and non-adherent cells removed by washing the biofilms twice with sterile ultra-pure water [14].

Quantification of total biomass by Crystal Violet (CV) (PARK inc, Northampton UK staining), after washing, biofilms were fixed with 200 µl of methanol, which was removed after 15 min of contact. The microtiter plates were dried at room temperature, and 200 µl of CV (1% v/ v) added to each well and incubated for 5 min. The wells were then gently washed with ultra-pure water. The absorbance of the obtained solutions was read in three separate experiments in a microtiter plate reader (Bio-Tek Synergy HT, Izasa, Lisbon, Portugal) at 620nm.

Antimicrobial assay (Disc diffusion assay): The crude extracts were dissolved in dimethyl sulfoxide (DMSO 1%), to a final concentration of 30 mg/ mL and sterilized through filtration by 0.45 µm Millipore filters. Antifungal tests were then carried out by the disc diffusion method [15] using 200 µl of suspension containing 1.10⁶ cfu.mL⁻¹ of yeast, spread on SDA medium. The discs (6 mm in diameter) were impregnated with 10 µl of extracts placed on the inoculated agar. Negative controls were prepared using DMSO (1%) used to dissolve the plant extracts. The inoculated plates were incubated at 37°C for 48 h.

Determination of Minimal Inhibitory Concentration (MIC) and Minimal Fungicidal Concentration (MFC)

The MIC and MFC determinations were measured according

to modified methods described by Sahin, et al. [16]. Leaf extract was diluted with same solvents to produce two-fold serial dilutions ranging from 0.5 to 32 mg/ ml. From each prepared solutions 1ml was added into test tubes containing 5 ml YPG broth. Yeasts suspensions (1 ml) prepared from 24 h YPG broth cultures and adjusted to 0.5 McFarland turbidity, were added to the tubes containing olive leaf extracts or solvent used as control. Test tubes were then incubated at 37°C for 48 h. Determination of the total viable cells was performed using YPG plate count agar, which were inoculated with serial dilutions and incubated at 37°C for 48h. Countable plates are those containing from 0 to 250 colonies [17]. The MIC₅₀ and MIC₉₀ of the test extracts were the lowest concentration of extracts that did permit only 50% and 10% respectively of cells to survive. The MFC was defined as the lowest concentrations that completely inhibit the growth of yeasts in YPG plates at 37°C for 48 h.

Determination of viability of *Candida glabrata* strains at sub-MICs of olive leaf: The test strains at a cell density of 1.10^6 cfu.mL⁻¹ were inoculated into test tubes containing 10 ml of YPG amended with sub-MICs (0.25 × MICs) of test agents for 24 h at 37°C. A control was run without test agents but containing yeast inoculum at equal cell density. Viable counts were obtained from the test and control solutions by plating 100 µL of 10-fold serial dilutions onto SDA plates and incubating at 37°C for 24 h.

Inhibition of virulence factors by olive leaves at sub-MICs

Inhibition of proteinase production: Briefly, BSA agar plates amended with sub-MICs (0.25 × MIC) of OLE and fluconazole were inoculated with 100 µL of *Candida glabrata* cells (1.10^6 cfu.mL⁻¹) and incubated at 37°C for 7 days. Proteinase production was determined in terms of the ratio of the diameter of the colony to the total diameter of colony plus zone of halo using the method described by Price, *et al.* [12]. The inhibition of proteinase production by olive leaves was determined as $R = (\text{Pz in treated sample} - \text{Pz untreated sample})$. A ratio = 0 indicated no inhibition of enzymatic activity and $R > 0$ indicated inhibition of proteinase activity. Each experiment was conducted three times with three replicates per experiment.

Inhibition of haemolysin production: *Candida glabrata* cells were grown in blood agar plates as previously described containing 0.25 × MIC of test agents for 4 days at 37°C. The solution without test agents was considered as untreated control. Each experiment was conducted three times with three replicates per experiment. The inhibition of hemolysin production by olive leaves extract was determined as $R = (\text{Pz in treated sample} - \text{Pz untreated sample})$. A ratio = 0 indicated no inhibition of enzymatic activity and $R > 0$ indicated inhibition of haemolysin activity.

Inhibition of phospholipase production: *Candida glabrata* cells were grown in egg yolk medium as previously described containing 0.25 × MIC of test agents for 5 days at 37°C. The solution without test agents was considered as untreated control. Each experiment was conducted three times with three replicates per experiment. The inhibition of phospholipase production by olive leaves extract was determined as $R = (\text{Pz in treated sample} - \text{Pz untreated sample})$. A ratio = 0 indicated no inhibition of phospholipase activity and $R > 0$ indicated inhibition of phospholipase activity.

- Pz untreated sample). A ratio = 0 indicated no inhibition of phospholipase activity and $R > 0$ indicated inhibition of activity.

Inhibition of biofilm formation by olive leaf extract at sub-MICs

Effect of olive leaf extract on biofilm formation: The effect of different sub-MICs of OLE and fluconazole (0.25 × MIC) on the ability of *Candida glabrata* to form biofilm was determined as previously described with some modifications. Volumes of 0.1 mL of test agents (2 × final concentrations) in YPG medium was added to each well of microtiter plates. Subsequently, 0.1 mL of standardized yeast cell suspension was added and plates were incubated at 37°C for 48h. Antifungal agent-free wells served as positive controls for biofilm growth. After incubation, the medium and non-adherent cells were removed from wells and washed three times with sterile PBS. Further biofilm formation was analyzed by CV reduction assay. Absorbance values were used to measure the inhibition of biofilm formation as follows: $(\text{mean OD}_{620} \text{ of treated well} / \text{mean OD}_{620} \text{ of untreated control well}) \times 100$.

Statistical analysis: All the experiments were performed three times with three replicates per experiment and data are expressed as mean ± standard deviation. Statistical significance of the differences was determined by using Student's t-test (SPSS version 13.0) and ANOVA test. Differences were considered significant at $p \leq 0.05$. All *in vitro* experiments were performed respectively at least three times.

Results

C. glabrata virulence factors: extracellular hydrolases and biofilm formation

We collected 61 *C. glabrata* isolates from blood samples, buccal and vaginal site. All isolates were tested for susceptibility to fluconazole. All isolates have a high susceptibility to Fluconazole (CMI₉₀ = 0.25 to 8 µg/ ml). Moreover three isolates showed *in vitro* resistant profile (CMI₉₀ > 64 µg/ ml).

We investigated in the present study *in vitro* virulence factors of 61 *Candida glabrata* isolates [Figure 1]. The production and quantitative expression of these factors in different *C. glabrata* isolates were recapitulated in Table1. All tested *C. glabrata* isolates were able to produce haemolysin with an average Pz mean (0.684 ± 0.118) and to form biofilm ($A_{620} = 0.3 \pm 0.42$), 98.3% of isolates were phospholipase producer with an average

Figure 1: Culture of isolates of *C. glabrata* in medium rich on egg yolk (10%) (A), on blood agar plate (5%) (B) and BSA (1%) (C). A white halo around the colonies indicate an enzymatic production.

Table 1: Level of phospholipase, haemolysin and protease production by *Candida glabrata* isolates from blood culture, vaginal and buccal sites

		Phospholipase activity (number of isolates/ rate of isolates)			Haemolysin activity (number of isolates/ rate of isolates)			Protease activity (number of isolates/ rate of isolates)		
		Blood culture (n = 35)	Vaginal (n = 21)	buccal (n = 5)	Blood culture (n = 35)	Vaginal (n = 21)	buccal (n = 5)	Blood culture (n = 35)	Vaginal (n = 21)	buccal (n = 5)
Level of enzymatic Production	Strong	16(45.7%)	7(33.3%)	2 (40%)	16(45.7%)	11(52.3%)	3(60%)	33(94.2%)	19(90.4%)	5(100%)
	Moderate	19(54.2%)	13(61.9%)	3(60%)	19(54.2%)	10(47.6%)	2(40%)	1(2.8%)	1(4.7%)	0
	Weak	0	0	0	0	0	0	0	0	0
	Null	0	1(4.7%)	0	0	0	0	1(2.8%)	1(4.7%)	0

Figure 2: The distribution of biofilm formation (A), haemolysin activity (B), phospholipase activity (C) and protease activity (D) among blood culture, vaginal and buccal clinical isolates.

production ($Pz = 0.69 \pm 0.13$) and 96.7% were protease producer with an average production ($Pz = 0.58 \pm 0.11$).

The average production for invasive *C. glabrata* isolates was ($Pz = 0.58 \pm 0.12$) for protease activity, ($Pz = 0.704 \pm 0.118$) for haemolysin activity and ($Pz = 0.69 \pm 0.126$) for phospholipase activity [Figure 2].

Relationship between the average Pz values of phospholipase activity, protease activity, hemolysin activity and the infection sites were demonstrated in figure 2. The One Way ANOVA test showed a non significant difference in haemolysin, phospholipase and protease activity between oral cavity, vulvovaginal and blood culture isolates ($p \geq 0.05$).

Biomass quantification results of the *in vitro* biofilm formation are shown in Figure 2A. The amount of biofilm formed in each case was estimated in triplicate and an average was calculated for each isolate. The amount of obtained biomass were (A_{620} , 0.503 ± 0.522) for invasive isolates, (Abs moy, 0.43 ± 0.23)

for vaginal isolates (Abs moy, 0.344 ± 0.13) for buccal isolates. For each strain, a small change in Abs (A_{620nm}) value was noted. It was noted that the total amount of biomass formed *in vitro* by invasive isolates was slightly higher than that formed by vaginal and buccal. However, this difference was proven insignificant (p value ≥ 0.05). The highest level of absorbance was observed for two invasive isolates 211BC and 362BC (Abs₆₂₀, 2.8 ± 0.19 and 2.1 ± 0.21 respectively) which formed a biomass amount high as 9 and 7 folds.

Olive Leaves Extract (OLE) and antifungal activity

In the present study, olive leaf extracts of *Chemlali* variety were screened for their activity against some *C. glabrata* isolates. A collection of isolates (*C. glabrata* ATCC 90020, 211BC, 636BC, 603BC, 362BC, 375VVC and 51VVC) were selected on the basis of being producers of an important amount of proteinase, phospholipase and/or haemolysin and biomass in polystyrene plate from two independent experiments. Selected isolates were inoculated in OLE at different concentrations (0, 0.5, 1, 2, 4, 8, 16, 32 and 64 mg/ ml). *C. glabrata* growth was dramatically decreased after treatment with OLE. Light microscopy images were captured at 1, 2 and 16 mg/ ml of 211BC isolate [Figure 3]. As evident in figure 3, *C. glabrata* cells were killed after 24h of incubation in 16 mg/ ml of OLE. Deduced MIC values were ranging from 2 to 8 mg/ ml and MFC values from 16 to 32 mg/ ml [Table 2].

Anti-virulence activity of olive leaf extract against clinical isolates of *C. glabrata*

OLE was further assessed for their anti-virulence activity at sub-MICs ($0.25 \times \text{MICs}$) against the strains *C. glabrata* ATCC90020, 211BC, 636BC, 603BC, 362BC, 375VVC and 51VVC. According to the Pz values, there was a significant decrease ($p < 0.05$) in the production of extracellular hydrolases by *C. glabrata* strains treated with and without OLE.

At 0.25 x MIC tested concentration, OLE was able to reduce *in vitro* proteinase production by an average of 55.87%, the phospholipase activity by 46.85% and haemolysin activity by 25.37% (Table 3). Maximal reducing activity was observed against *C. glabrata* ATCC90020 with 81.8% of protease activity and the minimal was almost 4% for phospholipase activity against 636BC isolates [Table 3].

As presented in Figure 4, the biofilm formation by *C. glabrata* isolates was significantly decreased in the presence of increasing concentration of OLE. Biofilm formation in both tested strains was reduced. At 0.25xMIC of OLE, an amount of 2.43 to 39.13% formation of biofilm was recorded in tested strains. Whereas fluconazole at 0.25 x MIC led to 47.62% formation of biofilm in *C. glabrata* 362HC, 52.7% of phospholipase activity, 53% of protease activity and 35.2% of haemolysin activity.

Discussion

Our data has highlighted the production of proteinase, phospholipase and haemolysin in the *C. glabrata* strains irrespective of their source of clinical isolation. The identification

Figure 3: Light microscopy (magnification, x40) images corresponding to the activity of Olive leaves extract against *Candida glabrata*. Negative control *C. glabrata* in YPG medium without any drugs at 24h(a) and *C. glabrata* in DMSO (1 %) at 24h (b). Treated cells with 1 mg/ ml (c), 2 mg/ ml (d) and 16 mg/ ml (e) of the *chemlali* variety olive leaves at 24h. *Candida* growth was dramatically decreased after treatment.

Table 2: The minimal inhibition concentration (MIC₅₀/ MIC₉₀/ MFC) of olive leaf extracts against some isolates.

Isolates n°	Source	MIC ₅₀ (mg/ml)	MIC ₉₀ (mg/ml)	MFC (mg/ml)
Reference strain	<i>C. glabrata</i> ATCC90020	4	8	32
211BC	Blood culture	0.5	4	32
603BC	Blood culture	0.5	2	16
51 VVC	vaginal	0.5	4	64
362BC*	Blood culture	0.5	8	32
636BC	Blood culture	0.5	4	8

*MIC (fluconazole) 362BC = 256 µg/ ml; all others isolates were susceptible; BC: Blood Culture; VVC: Vulvovaginal Candidiasis

Table 3: Comparative analysis of enzymatic activities and inhibition rate for treated and untreated with olive leaves extract in some isolates of *C. glabrata* susceptible or resistant to fluconazole.

Virulence factors	Isolates	Antifungal drugs			Student test
		0.25 MIC olive leaves extract	Without any drugs	Inhibition rates (%)	
Phospholipase activity (Pz)	362 HC	0.86	0.48	79.17	$P < 0.05$
	603 HC	0.89	0.64	39.06	
	211 HC	0.9	0.54	66.67	
	636 HC	0.86	0.83	3.61	
	<i>C. glabrata</i> ATCC90020	0.86	0.59	45.76	
Haemolysine activity (Pz)	362 HC	0.84	0.62	35.48	$P < 0.05$
	603 HC	1	0.78	28.21	
	211 HC	0.8	0.7	14.29	
	636 HC	0.8	0.58	37.93	
	<i>C. glabrata</i> ATCC90020	0.81	0.73	10.96	
Protease activity (Pz)	362 HC	1	0.71	40.85	$P < 0.05$
	603 HC	1	0.64	56.25	
	211 HC	0.8	0.64	25	
	636 HC	1	0.57	75.44	
	<i>C. glabrata</i> ATCC90020	1	0.55	81.82	

MIC (FCZ) ₃₆₂ = 256 µg/ ml; MIC (FCZ) ₆₀₃ = 2 µg/ ml; MIC (FCZ) ₂₁₁ = 0.25 µg/ ml; MIC (FCZ) ₆₃₆ = 1 µg/ ml; MIC (FCZ) _{ATCC90020} = 2 µg/ ml

Figure 4: Decreasing biofilm production by selected *C. glabrata* isolates treated with different concentrations of olive leaves extract.

of these virulence factors unique to *C. glabrata* could provide powerful insights into the pathogenic process and will hopefully reveal novel antifungal drug targets that can be exploited in the search for improved therapies [17]. Extensive research on virulence factors were focused on *C. albicans*. However, quite a few research studies refer to virulence factors production in *C. glabrata*. Most of them have agreed that only a few *C. glabrata*

isolates were phospholipase and protease producers and that *C. albicans* is considered as the most pathogenic species [18-20].

Chin, et al. studied the production of phospholipase in 37 strains of *Candida* and have shown positive activity in 75% of *C. albicans* isolates but no *C. glabrata* strain [18]. However, Ghannoum, et al. [21] showed that phospholipase activity is considered a rather important activity for candidemia in *C. glabrata*. During our work, all invasive and oral isolates and 95% of vaginal isolates produced phospholipases. Even though, Phospholipase activity does not vary with anatomical location but with geographical origin of isolates [22,23].

In the same way, 96.7% of isolates from different anatomical sites were protease producers. In contrast, Yamamoto, et al. [24] studied the secretory activity in 52 strains *C. glabrata* but no strain showed a proteolytic capacity. Seneviratne, et al. [25] evaluated the virulence attribute of *Candida* bloodstream isolates derived from Hong Kong and Finland and showed that proteinase activity from the finlandais *C. albicans* isolates was significantly higher than the Hong Kong isolates. The variability of virulence factors expression may be explained by possible evolutionary changes in the pathogenic potential of *Candida* depending on geographic regions [26].

Haemolysin is an enzyme that degrades the erythrocytes of the host, thereby releasing the iron for later use during invasive infections. In our study, all isolates have similar capabilities to produce haemolysin, and like to phospholipase and proteases, haemolysin appears to be an important virulence factor for all *C. glabrata* isolates. Rossoni, et al. tested the haemolysin activity of 50 strains of *Candida spp.* and found that all strains of *C. albicans* and 92% of *C. glabrata* isolates showed hemolytic activity and the level of haemolysin production was significantly higher in *C. glabrata* isolates [27].

All tested isolates showed the same capacity to produce protease, phospholipase and haemolysin and analysis of variance by multifactor ANOVA illustrated that enzymatic activity was not dependent of isolation site ($p \geq 0.05$). This observation was dissimilar to the reports given by previous authors whose have concluded that phospholipase and protease production may be used as one of the parameters to distinguish virulent invasive strains from non-invasive colonizers [22]. In fact, many papers confirmed that enzymatic activity of *Candida* isolates depends also to evolutionary changes that occur between different geographic origins [23-25].

Efficient treatment for fungal infections becomes very important as the frequency of life-threatening fungal diseases is increasing due to the progress in the treatment of critically ill patients whose immune status has been deteriorated. Therefore, the development of novel antifungal agents or strategies that may be effective against pathogens resistant to currently available antifungal drugs is of paramount importance. Considering this, we attempted to investigate concentration dependent effect of Olive Leaves Extract (OLE) against growth and virulence factors production in the fluconazole-resistant or susceptible strains of

C. glabrata. In our study, similar pattern of biofilm formation and enzymes production was also recorded for test leaves and fluconazole drug against *C. glabrata* 362BC. Comparably to several papers, OLE provides a stronger activity against fungi and parasite [6-8,28-31]. Masoko and makgapeetja when tested O. Africana extracts against *C. albicans* and *Cryptococcus neoformans* demonstrated that both were sensitive to the plant extracts with average MIC values of 0.37 and 0.30 mg/ ml [28].

In our study, OLE exhibited MIC value in the range of 2-8 mg/ ml against selected isolates. The MIC is greater than that previously described for *C. albicans* [28]. However, many authors confirmed that the biological activity of plant extract depends on the plant variety and/or extraction procedure which determines the success of isolating compounds from plant material [28,29]. Indeed, Markin et al. reported that OLE extracted with water succeeded in elimination of some GRAM-bacteria such as *Escherichia coli*, *Pseudomonas aeruginosa* and *Klebsiella pneumonia* with Minimum Bactericidal Concentration (MBC) varied between 0.13% to 0.6% and also some yeast such *C. albicans* with Minimum Fungicidal Concentration (MFC) equal to 15% [30]. Moreover, Karygianni, et al. [31] demonstrated that OLE extracted with acetone eradicated microbial strains with MIC value between 0.07 mg/ ml for *Streptococcus oralis* and 10 mg/ ml for *C. albicans* and *Escherichia coli*. In our study, we used methanol to extract compounds from olive leaves which was proven the most polar solvent according to several publications [29,32]. The slight difference between the activities of the extracts from many studies could be attributed to the different chemical profile due to plant variety and/or the extraction solvent. Our extract inhibited not only the growth of *C. glabrata* isolates but also the production of virulence factors essentially biofilm formation, production of proteases, phospholipase and haemolysin ($p \leq 0.05$).

Conclusion

We conclude that *C. glabrata* isolated from Sfax University Hospital (CHU Habib Bourguiba) presented a high virulent power for studied factors and these activities did not depend on the sampling sites but may be on possible evolutionary changes that may occur between different regions of the world. These results suggested also that extract olive leaves evaluated here is a potential therapeutic drug for the treatment of *C. glabrata* infection. OLE affects not only cellular growth but also cellular adhesion on a solid support and the production of virulence enzymes by *C. glabrata* isolates. Novel therapeutics that target virulence rather than simply *in vitro* cell growth is of particular interest firstly because of the low sensitivity of *C. glabrata* isolates to azole antifungal drugs and secondly because biofilm formation remains a crucial problem especially in hospitalized patients.

Declarations

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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