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Garlic-induced Proteomic Change, Anti-biofilm and Antifungal Susceptibility of *Candida albicans*

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Biofilm; *Candida albicans;* minimum biofilm inhibitory concentration; planktonic; protein pattern.

ABSTRACT

Background*:** The increasing azole-resistance among *Candida albicans* isolates worldwide justifies the search for newer antifungal natural agents, such as garlic. This study aimed to explore the antifungal and antibiofilm activity of garlic extract on *Candida albicans*, and the associated polymorphism of protein bands. Results were compared to fluconazole. The minimum inhibitory concentration (MIC) of garlic extract and fluconazole was done for 19 *C. albicans* clinical isolates using the micro broth dilution method. The antibiofilm activities of garlic and fluconazole were studied, and the minimum biofilm inhibitory and eradication concentrations (MBIC and MBEC, respectively) were measured. *C. albicans* protein expression patterns were assessed using protein electrophoresis. ***Results*:** Garlic had a better inhibitory effect on planktonic cells (lower MICs) compared to fluconazole (94.4% *versus* 77.7%, respectively). In contrast, fluconazole had a better anti-biofilm activity compared to garlic (88.9% and 72.2%, respectively). Garlic inhibited and eradicated the formation of biofilm in 72.2% and 61.1% of samples, respectively, while 88.9% of isolates were equally inhibited and eradicated by fluconazole. The median number of protein bands of untreated planktonic isolates was nine bands, while garlic-treated and fluconazole-treated planktonic isolates produced significantly more bands [median of 16 bands ($p< 0.001$) and 14 bands ($p= 0.003$), respectively]. ***Conclusions: The proteomic changes associated with antifungal activity caused by garlic exposure highlight its potential role as a natural antifungal and anti-biofilm agent. Paradoxical regrowth of cells suggests a fungistatic rather than fungicidal activity.

INTRODUCTION

Candida species are natural commensals of the human microbial flora, and it is considered opportunistic pathogen (Hallen-Adams and Suhr 2017). Studies on *Candida* infections in some countries showed that *Candida albicans* (*C. albicans*) was the most commonly isolated species with prevalence ranging from 22.3% to 60%. The misuse of antifungal agents has led to the growing antifungal resistance of *C. albicans* and the rise of non- albicans *Candida* species (Kmeid, Jabbour *et al.*, 2020).

Fluconazole resistance in *Candida* spp. has been reported increasingly in *C. albicans*, and requires the search for new therapeutic options. Biofilms are a barrier to treatment as they are often resistant to antifungal drugs. Therefore new antifungal agents should be tested for their effects on planktonic as well as biofilm-inhibiting and eradicating abilities (Berkow and Lockhart 2017).

Many studies have reported the antifungal activity of natural products (including garlic) against *Candida* species (Begnami, Duarte *et al.,* 2010). Garlic possesses antibiotic, anticancer, antioxidant, immunomodulatory, anti-inflammatory, and hypoglycemic properties (Harris, Cottrell *et al.,* 2001). The antifungal properties of garlic are due to the presence of sulfur compounds, mainly allicin, produced after the crushing and chopping of garlic bulbs. Allicin has a potent antifungal activity as it interferes with cellular metabolism by inhibiting thiol-containing amino acids and proteins (Ankri and Mirelman 1999). Also, allicin was found to prevent fungal spore formation, lipid synthesis, and hyphal growth (Low, Chong *et al.,* 2008). Proteomics enables studying states of cellular growth and how cells change in response to environmental factors (Bhadauria, Zhao *et al.,* 2007). Therefore, it is essential to identify proteins, especially those sharing low or no homology with human proteins, as they represent a potential target for drug or vaccine development (Champer, Ito *et al.,* 2016). One-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (1D, SDS-PAGE) is an efficient technique for analysis microbial proteins in different micro-organisms and it has been used as a taxonomic tool for typing based on microbial proteins' characters (Scarcelli, Costa *et al.,* 2001). Hence, this study aimed to study the anti-*Candida* effect of garlic, compared to fluconazole. Special emphasis was put on the effect of garlic on protein polymorphism of both planktonic and biofilm cells.

MATERIALS AND METHODS

This cross-sectional experimental study was conducted in the Microbiology laboratory of the High Institute of Public Health, Alexandria University. The study included 18 clinical *C. albicans* isolates in addition to one ATCC strain (#90028). Strains were identified by Vitek 2 compact® system (Biomerieux, France). The sample size was based on a previous study, in which the mean minimum inhibitory concentration of garlic was found to be 23.18 mg/ml with a standard deviation of 0.129 mg/ml (Hamdi and Khodavandi 2016). Using a precision of 0.06 and 95% confidence interval, the minimum sample size required was 18 clinical isolates of *C. albicans* (Sergeant 2018). For garlic extract preparation, garlic bulbs (Egyptian "Baladi" cultivar), which are the most common commercial garlic cultivars in Egypt were purchased from local markets in Alexandria. Bulbs were washed with sterile saline then air-dried. A weight of 157.36 gm of clean air-dried bulbs was blended then soaked in 70 ml of absolute ethanol for 24 hours (hrs) in a sterile glass bottle. The bottle was shaken vigorously to allow for the proper extraction of active ingredients. The crude extract was then sterilized by syringe filtration using a 0.22 μm syringe filter. The filtered extract was then stored in the refrigerator at 4°C (Tagoe, Nyarko et al. 2011). Antifungal activities of garlic and fluconazole (Sedico, Egypt) were estimated as minimum inhibitory concentration (MIC) by microbroth dilution test according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI), (M27-A3)((CLSI) 2017). Garlic extract and fluconazole concentrations ranges were (112.4 – 0.21mg/ml) and (64 – 0.125μg/ml), respectively. The final concentrations were prepared using RPMI 1640 medium with L-glutamine, without sodium bicarbonate (Sigma-Aldrich, Germany -R 6504) buffered to pH 7.0 with 0.165 M 3-N-morpholino propane sulfonic acid (MOPS)- (Sigma-Aldrich, Germany-M3183). Methodology for

biofilm formation and inhibition was adapted from previous studies (Stepanović, Vuković *et al.* 2007, Turan and Demirbilek 2018). *Candida* suspensions were prepared from overnight cultures in 5 mL yeast nitrogen base (YNB) at 37º C for 18 hrs. The cells were then centrifuged at 2.000 g for 10 minutes, and the supernatant was discarded. The sediment was re-suspended in PBS, and the tubes were vortexed for 30 seconds. Cell washing was repeated twice, and cell densities were adjusted spectrophotometrically at 10^6 cells /ml in YNB. Each well was inoculated with 100 µl of *Candida* suspension and incubated for 24 hrs at 37° C for biofilm establishment. After that, wells were washed twice with PBS and then heat-fixed in a hot air oven at 56º C for 45 minutes. The wells were then stained with 150 µl 0.3% aqueous crystal violet (CV) solution for 30 minutes. Stained wells were aspirated, washed twice PBS, and then de-stained with 150 µl 33% acetic acid for 30 minutes. Then, 100 µl of de-staining solution were transferred to a new 96- well flat-bottom microtiter plate. Two wells were preserved as blanks to be inoculated later with 100 µl acetic acid only. Absorbance values were measured with a microtiter plate reader (BIO-TEK, ELx800, USA) at 570 nm (de Alteriis, Maselli et al. 2018) .

MBIC Determination*:*

Minimum biofilm inhibitory concentration (MBIC) is defined as the lowest concentration of fluconazole or garlic that reduced biofilm formation by 50% (Girmenia, Tuccinardi et al. 2000, Teh, Nazni *et al.* 2017) compared to the negative (untreated) control. For MBIC determination, more concentrated MIC solutions of garlic/ fluconazole were additionally prepared (2MIC, 4MIC, 8MIC) in sterile 96-well flat-bottomed microtiter plates. The wells were simultaneously inoculated with *Candida* suspension (as mentioned above) and incubated at 37 ºC for 24 hrs (Gong, Seo *et al.*, 2019). Biofilm was fixed, stained, and measured as mentioned above.

MBEC Determination:

For MBEC determination, biofilms were allowed to mature in the second set of plates then the plates were aspirated and washed with PBS. One hundred µl of garlic and also for fluconazole were added and the plates were incubated for 24 hrs at 37°C then, the agents were discarded and the plates were washed with PBS and 200 µl of fresh YNB without garlic or fluconazole were added to all wells, and incubated for 24 hrs. Biofilm cells were scraped from the wells using sterile tips. Then, 100 µl from each well were inoculated on SDA plates and incubated for 24 hrs. Colonies were counted using the spread plate count technique.

Protein Extraction and Fragmentation:

For determining the effect of garlic on protein patterns of planktonic cells and biofilms, total cellular proteins were extracted from planktonic isolates (at MIC values) and mature biofilms (at MBIC values) of *C. albicans* before and after exposure to garlic and fluconazole. Protein extraction was performed according to Laemmli et al. (Laemmli 1970). Protein extraction was carried out by TriFast™ kit (Peqlab, VWR company, UK- Catalogue no. 30-2030). Samples were electrophoresed using protein electrophoresis apparatus. For each sample, 30 µg proteins were loaded and electrophoresis was performed at 75 volts on stacking gel followed by 125 volts for approximately 2 hrs. Gels were stained by 0.1 % Coomassie blue R- 250 stain (Himedia, India) for 2 hrs, then de-stained with a solution (1:3:6) of glacial acetic acid; methanol; and water, respectively. Protein expression was noted in terms of the number of protein bands after fragmentation as well as polymorphic bands. Protein polymorphism was calculated as the number of newly appearing bands divided by the total number of protein bands in each isolate.

RESULTS

At the selected ranges for susceptibility testing in our study, 94.4% of isolates were sensitive to garlic extract, with MIC ranging from 0.87 to 14 mg/ml (Table1). The majority of isolates (77.7 %) were susceptible to fluconazole, with MIC ranging from 0.125 - 8 µg/ml.

The majority of *C. albicans* isolates (n=12, 66.7%), as well as the ATCC strain, were weak biofilm-producers; two isolates were intermediate-producers (11.1%), while 22.2% of (n=4) were strong producers. Garlic and fluconazole reduced the formation of biofilm in 72.2% and 88.8% of samples, respectively. Assessing the antifungal effect on biofilms was accomplished by measuring MBIC and MBEC (Table 2). The tested MBIC for garlic extract ranged from 0.87 to 112.4 mg /ml, while it ranged from 0.125 - 64 µg/ml for fluconazole. In 38.8% of garlic-treated isolates (n=7), the MBICs were the same as the MICs for the same isolates, while 55.5% (n =10) of fluconazole-treated isolates had MBICs identical in value to the MICs of the same isolates.

Garlic extract eradicated the mature biofilm in 61.1% of isolates, while fluconazole eradicated the mature formed in 88.9%, of isolates. For biofilm eradication, the MBEC ranged from 0.87 to 224.8 mg/ml after garlic exposure and 0.125 -128 µg/ml for fluconazole. Furthermore, it was found that 66.7% of isolates exposed to garlic had higher MBEC than their MIC values, as opposed to only 33.3% of fluconazole-treated isolates, which had higher MBEC values than their MICs. Thus, there was a variable response to garlic and fluconazole between isolates.

Paradoxical growth was noted during the determination of MBEC of garlic and fluconazole, with an isolate-to-isolate variable manner. Interestingly, biofilm-garlic-treated isolates numbers: 1, 2, 3, 4, 8, 9, 10 and the ATCC strain (no. 11) samples showed paradoxical re-growth of cells on SDA at concentrations higher than their MBEC. Similarly, paradoxical growth was seen in fluconazole-treated biofilms of isolates no. 2, 4, 6, 8, 12, 13, 15, 16, and 18 at concentrations exceeding their MBEC values.

In planktonic *C. albicans* isolates, the effect of garlic extract and fluconazole on protein patterns (Figs. 2 and 3) was statistically different ($p< 0.001$, $p =0.003$, respectively) than their untreated counterparts (table 1, figures 1). Garlic extract caused more prominent protein fragmentation in *C. albicans* planktonic isolates compared to fluconazole ($p< 0.001$) (Table 1, Figs. 3 and 4). A dense band appeared approximately at 49-50 kDa in 88.9%, of isolates after garlic treatment of planktonic isolates (Fig 2). A dense band appeared approximately at 49-50 kDa in 72.2% of isolates after fluconazole treatment of planktonic isolates (Fig. 3). The number of protein bands from untreated biofilms was similar to that of untreated planktonic cells (median=9 and 11, respectively), (Tables 1 And 2).

Opposite to the pattern of untreated samples, biofilm samples showed a statistically higher number of protein bands in garlic and fluconazole-treated *C. albicans* biofilms compared to untreated biofilms ($p = 0.027$, $p = 0.008$, respectively) (Table 3). However, the difference between the two agents was not statistically significant. Also, it was noticed that garlic caused a decrease in the number of protein bands (isolates no. 12, 13, and 14) but not in their planktonic counterparts (figure of protein electrophoresis not shown).

Similarly, fluconazole caused a decrease in the number of bands both in planktonic and biofilm cells compared to their untreated counterparts (isolates no. 10, 16, 8, 13, and 18, respectively). The polymorphic protein variation induced by fluconazole (median =51.9%) was higher than that induced by garlic (median=13.4%) in the case of planktonic cells ($p< 0.001$) (Table 1). Regarding polymorphism in biofilms, garlic extract and fluconazole produced 26.0% and 21.8% polymorphic bands, respectively ($p= 0.006$) (Table 2).

Table 1. MIC, MBIC, and MBEC values of garlic (mg/ml) and fluconazole (µg/ml) against 18 *C. albicans* clinical strains and one *C. albicans* ATCC (90028) strain.

MIC	**Garlic (mg/ml) (n = 17)**	**Fluconazole (µg/ml) (n = 13)**
Min. – Max.	0.87 – 14.0	0.125 – 8.0
Median	3.50	0.50
ATCC 90028 (no.11)	7 (S)	0.125 (S)
MBIC	**(n = 13)**	**(n = 16)**
Min. – Max.	0.87 – 112.4	0.125 – 4.0
Median	3.50	0.75
ATCC 90028 (no.11)	7	>1
MBEC	**(n = 12)**	**(n = 16)**
Min. – Max.	0.87 – 224.8	0.125 – 128.0
Median	3.5	1.0
ATCC 90028 (no.11)	7	>1
p-value	0.459	0.205

Table 2: Number of protein bands and percentage polymorphism in planktonic cells of *C. albicans* before and after exposure to garlic extract and fluconazole.

Isolate number	**No. of bands of planktonic isolates**			**% Polymorphism of planktonic isolates**	
	Untreated	**Garlic-treated**	**Fluconazole-treated**	**Garlic-treated**	**Fluconazole-treated**
1	9	14	10	21.4	30
2	9	16	10	6.2	40
3	8	13	13	30.7	46.1
4	11	16	12	12.5	33.3
5	15	18	10	27.8	30
6	9	18	10	16.7	30
7	9	16	12	18.7	50
8	10	18	10	11.1	80
9	10	16	10	0	70
10	9	16	9	6.2	55.5
11(ATCC)	9	16	12	6.2	41.7
12	6	14	13	14.3	53.8
13	7	18	10	11.1	50
14	5	18	10	5.5	60
15	10	15	14	20.0	85.7
16	10	14	8	14.3	50
17	9	15	13	6.7	61.5
18	7	16	14	12.5	57.1
19	9	16	13	20.0	69.2
Median	9	16	10	13.4	51.9
p- value	$p_1 = <0.001^*, p_2 = 0.003^*, p_{3<} 0.001, p_4 < 0.001^*$			$p_5 < 0.001^*$	

p: p value for comparing between the number of bands in studied groups:
p1: p value for comparing number between untreated and garlic-treated samples
p2: p value for comparing between untreated and fluconazole-treated samples
p3: p value for comparing between garlic and fluconazole-treated samples
p4: p value for comparing between untreated, garlic and fluconazole-treated samples
p5: between percentages polymorphism of garlic and fluconazole (planktonic samples) *: Statistically significant at $p \leq 0.05$

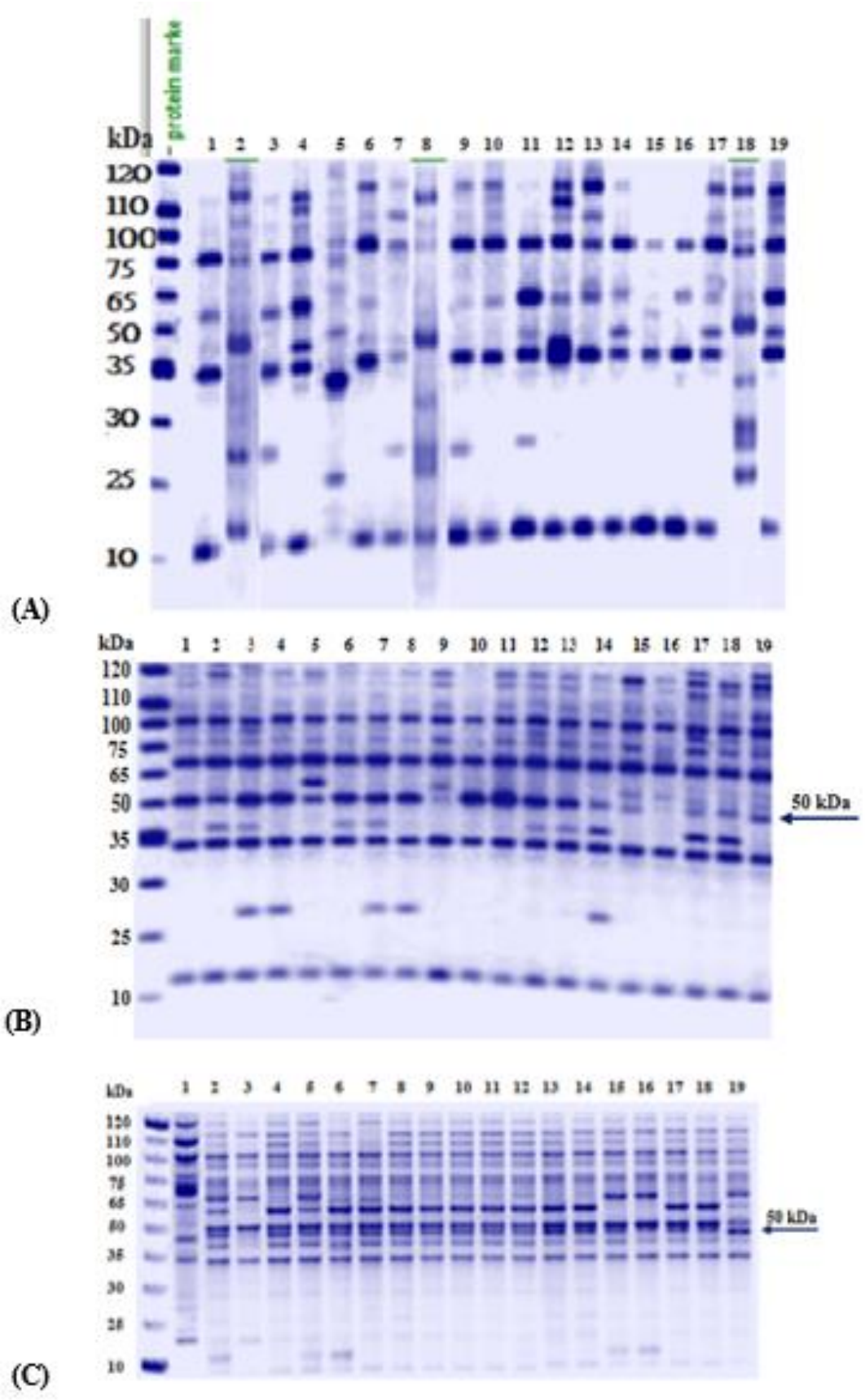

Fig. 1: Protein patterns of *C. albicans* planktonic isolates. (**A**): untreated, (**B**): garlic-treated, (**C**) fluconazole-treated**.**

Table 3: Number of protein bands and percentage polymorphism of *C. albicans* biofilms before and after exposure to garlic extract and fluconazole

Isolate Number	No. of bands of biofilm isolates			% Polymorphism of biofilm isolates	
	Untreated	Garlic-treated	Fluconazole-treated	Garlic-treated	Fluconazole-treated
1	8	13	14	15.3	14.2
2	12	12	14	16.6	21.4
3	9	11	12	27.2	8.3
4	7	10	10	40	20
5	7	11	10	18.1	40
6	11	12	12	25	33.3
7	10	14	10	7	30
8	12	12	10	25	20
9	8	15	13	33.3	38.4
10	7	13	11	15.3	18.1
11(ATCC)	11	12	11	33.3	27.2
12	12	11	13	45.5	38.4
13	12	10	9	50	22.2
14	11	9	15	33.3	20
15	11	14	15	21.4	20
16	12	12	15	33.3	40
17	12	13	14	45.4	35.7
18	13	12	12	30.7	16.6
19	12	12	12	20	33.3
Median	11	12	12	26	21.8
p- value	$p_1 = 0.027^*, p_2 = 0.008^*, p_3 = 0.897,$ $p_4 = 0.006^*$			$p_5 = 0.006^*$	

p: p value for comparing between the number of bands in studied groups:

p1: p value for comparing number between untreated and garlic-treated samples

p2: p value for comparing between untreated and fluconazole-treated samples

p3: p value for comparing between garlic and fluconazole-treated samples

p4: p value for comparing between untreated, garlic and fluconazole-treated samples

p5: between percentage polymorphism garlic and percentage polymorphism fluconazole in biofilm samples

*: Statistically significant at $p \leq 0.05$

DISCUSSION

Similar to our findings, Diba et al., reported *in vitro* antifungal properties of garlic. The antifungal properties of garlic are attributed to inhibition of fungal growth, inhibition of proteins, lipid, and nucleic acid synthesis (Champer, Ito *et al.*, 2016). Inhibition of ergosterol synthesis by garlic was also reported (Low, Chong et al. 2008). The antifungal activity of garlic was also reported in a mouse model of *C. tropicalis* systemic infection (Diba and Alizadeh 2018). In our study, the MIC for fluconazole ranged from 0.125 - 8 µg/ml. Similar values were reported in other studies (Gao, Wang *et al.*, 2016, Pesee, Angkananuwat *et al.*, 2016) . Similar to our results, Khodavandi *et al.*, noted that both allicin (not garlic extract) and fluconazole showed different MICs that ranged from 0.05 to 12.5 µg /ml and 0.25 to 16 µg/ ml, respectively for different *C. albicans* isolates (Khodavandi, Alizadeh et al. 2011). This variation in MIC values between studies is probably due to methodological issues such as differences in garlic species between countries, preparation method, and time until use.

In the present study, higher concentrations of the tested agents were used for MBIC and MBEC determination. This was done since biofilms are expected to be

more tolerant to antifungal drugs than planktonic cells, thus requiring higher concentrations. As MIC refers to the antifungal effect on planktonic cells, the MBIC and MBEC reflect the antifungal effect on cells embedded within the biofilm matrix (Gong, Seo *et al.*, 2019).

In 38.8% of garlic-treated isolates and 55.5% of fluconazole-treated isolates, MIC and MBIC were the same. This might indicate that antibiofilm activity was due to the antifungal effect of the tested agents on the sessile cells themselves rather than the biofilm structure, denoting a probably lesser role of extracellular matrix in those particular strains.

In another study, allicin-treated cells significantly reduced biofilm growth compared to fluconazole-treated and growth control cells. Moreover, allicin was shown to down-regulate the expression of *HWP1*, which is a gene involved in biofilm formation (Khodavandi, Harmal *et al.*, 2011). However, in our study, garlic extract was used rather than allicin. This anti-biofilm activity of garlic renders it a promising agent for inhibiting biofilm formation either on biogenic or non-biogenic surfaces. Said et al. (2020) reported that vaginal strains of *C. albicans*, when treated with fresh garlic extract, showed a reduction in *SIR2* expression in all strains.

In contrast, *ECE1* expression was up-regulated in isolates from patients unresponsive to garlic therapy. In addition, they reported that the garlic effect on biofilm was strain-dependent. The genetic impact of garlic on *C. albicans* denotes its powerful effect as an antifungal agent (Said, Watson *et al.* 2020).

Eradication of *C. albicans* mature biofilms formed on different medical devices is vital for eliminating *Candida* infections. In the present study, fluconazole had a better biofilm-eradicating ability than garlic (88.9% and 61.1%, respectively). In addition, around two-thirds of garlic-treated isolates had higher MBEC than their MIC values, as opposed to only one-third of fluconazole-treated isolates, which required higher concentrations of fluconazole for biofilm eradication than their MIC values. To the best of our knowledge, scarce data is available on the biofilm-eradicating abilities of garlic extract on *C. albicans*.

The paradoxical growth of certain garlic-treated and fluconazole-treated isolates on SDA at concentrations higher than their MBEC might denote a fungistatic rather than the fungicidal effect (Berkow and Lockhart 2017). A study reported that, despite inhibiting one cell wall component, another polymer is produced for compensation at higher concentrations of caspofungin. In addition, it was found that in *C. albicans*, biofilm persister cells show resistance to fluconazole, while the same cells grown in non-biofilm planktonic culture are sensitive to fluconazole (Cavalheiro and Teixeira 2018). This could justify the behavior of the isolates in the current study, which resisted higher concentrations of garlic and fluconazole above the MBEC level. A similar finding was observed by Wu et al. (2020), who conducted a study on biofilm formed by *C. albicans* in the vaginal epithelium of mice. They isolated antifungal- tolerant subset of cells called persister cells, which were not eradicated by high concentrations of antifungals (Wu, Zhang *et al.*, 2020). These results come in agreement with those of the current study.

Despite the long history of applying SDS-PAGE in scientific research, its use in evaluating microbial protein expression in response to treatment to date is limited. *Candida* proteomics has been studied in the planktonic state. However, studies on *Candida* biofilms are limited (Said, M. M., *et al.*, 2020). Studying protein fragmentation patterns helps explore the mechanism of action of drugs and explored antimicrobial agents. Other methods for studying microbial proteomics include the utilization of mass spectrometry, as in electron spray ionization (ESI) and matrix-assisted laser desorption/ionisation - time of flight mass spectrometry (MALDI-TOF), using peptide mass fingerprinting (Webster J 2012, Champer, Ito *et al.*, 2016).

Garlic extract caused more prominent protein fragmentation in *C. albicans* planktonic isolates compared to fluconazole ($p< 0.001$) (table 1). The increase in the number of protein bands may reflect the response of *C. albicans* planktonic isolates to the applied stress, which in this case would be the exposure to garlic or fluconazole. A study reported that fluconazole caused up-regulation of certain *C. albicans* proteins related to stress functions (Kustos, Nyul et al. 2006). According to Hanina *et al*. (2011), the appearance of new protein bands could be due to the synthesis of chaperones and signal transduction cascades that enable withstanding harsh stressful conditions (Hanina M. N. and Abdul Jalil A. K 2011). This justification could also be applied to both fluconazole and garlic in the current study (with a stronger effect of garlic), which showed an overproduction of protein bands. In the current study, the frequent appearance of a new protein-dense band at 49-50 kDa in 88.9%, 72.2% of isolates after garlic and fluconazole treatment of planktonic isolates (figure 1) denotes physical stress response in these isolates. Identifying newly appearing proteins might facilitate finding new drug targets. However, the identity and function of those new proteins were not addressed in the current study.

Due to the role of *C. albicans* biofilms in infections, proteomic analysis was previously used to study biofilm protein expression and identify possible drug targets (Vediyappan and Chaffin 2006). In the present study, the number of protein bands from untreated biofilms was similar to that of untreated planktonic cells (median=9 and 11, respectively, tables 1 and 2). This is similar to the results of Vediyappan et al. (2006). On the other hand, a study on *Pseudomonas aeruginosa* showed that its planktonic cells fragmented into more proteins bands than their biofilm counterparts did (Koziróg, Otlewska et al. 2018). As reported in other studies, this could be because carbohydrates constitute 40% of extracellular matrix composition rather than proteins (Cavalheiro and Teixeira 2018). In contrast, other studies recovered more biofilm proteins than planktonic proteins in *Pseudomonas aeruginosa* (Shafiei, Abdi-Ali *et al.*, 2017). The differences between biofilm and planktonic proteins profiles between studies could be due to different techniques (as 1D or 2D – SDS-PAGE) and different environmental conditions as pH and extraction methods (Koziróg, Otlewska *et al.*, 2018).

The decrease in the number of protein bands in biofilms could be due to the proteolytic activity of garlic on biofilm components or due to fungal death, as reported in similar studies (Peng, Kang *et al.*, 2015). Thus, the disappearance of protein bands in response to garlic in the current study could be due to the destruction of some fungal proteins. Similarly, fluconazole caused a decrease in the number of bands both in planktonic and biofilm cells compared to their untreated counterparts (isolates no. 10, 16, 8, 13, and 18, respectively). Thus, although the proteins produced were not identified in the current study, the decrease in the number of bands in the case of fluconazole could be due to the disintegration of cellular structural proteins.

To our knowledge, protein polymorphism was not investigated in response to garlic or fluconazole in *C. albicans* in previous studies. However, it was investigated while studying the antibacterial effect of ginger on some resistant bacteria (Attiya Mohamedin 2018). In the current study, the higher polymorphic protein variation induced by fluconazole (median =51.9%) compared to garlic (median=13.4%) in the case of planktonic cells ($p<0.001$) might imply that the antifungal effect of garlic on planktonic cells might occur through other mechanisms other than protein-related pathways. However, in planktonic isolate no. 9, polymorphism was absent (0%) despite the increase in the number of bands after exposure to garlic (Table 1). This also could be due to the degradation of the polymorphic proteins in this isolate into smaller polymorphic molecular weight proteins that the protein marker range could not detect.

Biofilms not only require higher antifungal concentrations for eradication but also for inducing polymorphic variation in their proteins as well. The polymorphism percentages, shown in tables 1 and 2, indicate that the effect of fluconazole on planktonic cells was more evident than on their biofilm counterparts.

Limitations:

Further studies on the antifungal effect of garlic and its mechanism of action are required. A limitation of this study is the use of whole extract rather than the active compound (alllicin), thus it was difficult to attribute the antifungal action witnessed in the study to a particular compound. Using isolated compounds from garlic would help explore the exact mechanism of antifungal action for each garlic isolated component. Also, the identification of the new protein bands and polymorphic ones was also not carried out in our study and would have been a valuable addition to our work.

Conclusions

Garlic extract possesses antifungal activity and is capable of reducing and eradicating *C. albicans* biofilms. Antifungal agents act at varying potencies depending on the state of the fungal cell (planktonic *versus* biofilm). Both garlic and fluconazole had a strain-dependent antifungal activity. Fluconazole induced more significant changes in protein fragmentation patterns of *C.albicans* planktonic isolates compared to ethanolic garlic extract.

List of Abbreviations:

NAC: non- albicans *Candida*, SDS: sodium dodecyl sulfate; MOPS: M 3-N-morpholino propane sulfonic acid; MIC: minimum inhibitory concentrations; MBIC: minimum biofilm inhibitory concentration; MBEC: minimum biofilm eradication concentration; *C. albicans*: *Candida albicans*; (MALDI-TOF) matrix-assisted laser desorption/ionisation - time of flight mass spectrometry.

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