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# Effects of fresh garlic extract on *Candida albicans* sessile cells, biofilms and biofilm associated genes, Flo-8 and Ndt80

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## ABSTRACT

*Candida*-associated nosocomial infections are a persistent problem which has been steadily increasing over the years. The emergence of antimicrobial resistant strains has narrowed the spectrum of effective drugs that can be utilised and indicated the need for alternative therapeutics. Garlic is a spice often studied for its unique characteristics. Most of its antimicrobial properties have been attributed to the presence of allicin, which makes up more than 70% of thiosulfinates extracted from garlic. The present study aimed to determine the effects of fresh garlic extract (FGE) towards the expression of two major biofilm genes, Flo-8 and Ndt80 produced by *C. albicans* that enable the morphogenesis of yeast from planktonic cells to biofilm. Minimum concentration required for FGE to inhibit *C. albicans* was determined through agar well and disc diffusion assay. Effect of FGE towards preformed *C. albicans* biofilms was evaluated at ¼× and ½× inhibitory concentrations of FGE and Amphotericin B, respectively by XTT assay. RNA from FGE-treated *C. albicans* was extracted, reverse transcribed and analysed by Real-Time Polymerase Chain Reaction (RT-PCR) to determine FGE effects towards the expression of Flo-8 and Ndt80. Inhibitory concentration of FGE was determined to be 100 mg/mL. Both genes appeared to be upregulated in the presence of FGE with a higher upregulation noted with Flo-8 (67.34 and 30.20-folds) when compared against Ndt80 (5.55 and 1.79-folds) at 5 mg/mL and 10 mg/mL, respectively. It is surmised that *C. albicans* upregulated these genes as a survival mechanism in the presence of FGE. The findings from this study indicate that FGE could inhibit *C. albicans* growth and biofilms. Further research is required to determine the minimum inhibitory concentration (MIC) through broth microdilution and to understand the mechanism behind this phenomenon before it can be utilised as a potential therapeutic to combat *Candida*-associated nosocomial infections.

***Keywords:*** *Fresh garlic extract; Candida albicans; biofilm and antifungal*

## INTRODUCTION

Garlic is a common household spice with many health benefits (Majewski, 2014) including antimicrobial properties. Its inhibitory action has been described as a result of a combined effect by several compounds such as allicin, ajoenes and diallyl disulfide (DADS) (Casella et al., 2012). A study to determine the effects of fresh garlic extract (FGE) against *Candida albicans* ATCC 14053 demonstrated a suppression of the hyphae production and reduced expression of the gene, *SIR*2 (Low et al., 2008). Allicin (diallyl thiosulfinate) is a sulfur-containing organic compound that makes up a large portion (~70% w/w) of thiosulfinates found in garlic (Rybak et al., 2004; Salehi et al., 2019). Previous studies have indicated that allicin is a promising

alternative therapeutic that has a lower risk of triggering resistance due to its mechanism of action (Jabar & Al-Mossawi, 2007).

*Candida* sp. particularly, *C. albicans* is the leading cause of nosocomial infections. This genus is a common opportunistic commensal of the human body often detected in the gastrointestinal tract, oral and vaginal tract. Its dimorphic nature contributes to its pathogenic nature as it enables this microorganism to switch from yeast to hyphal form under optimum conditions such as in immunocompromised individuals (Kim & Sudbery, 2011). Similarly, its ability to form biofilms enable it to adhere to surfaces and withstand harsh treatments, which inevitably gives rise to resistant strains (Nobile & Johnson, 2015).

Previous studies on the genetic mechanisms behind the biofilm formations of *C. albicans* identified six master regulators; Bcr1, Brg1, Efg1, Ndt80, Rob1 and Tec1 that are responsible for regulating the expression of over a thousand genes in matured (48 h) *C. albicans* biofilms by binding to their upstream intergenic regions. Three additional regulators, Flo-8, Rfx2 and Gal4 were later noted to be an integral part of the *C. albicans* transcriptional network as they were bound to at least one of the six master regulators (Fox et al., 2015). All six master regulators including Flo-8 are positive regulators that play a role in biofilm development. Interestingly, these transcriptional regulators could exert control over similar genes as the other core regulators in the network as well as genes that are unique to them, which suggest the existence of an intricate coordination system (Nobile et al., 2012). Flo-8 encodes a transcription factor that regulates hyphal development and hyphal-specific gene expression, and plays a major role in biofilm formation as its deletion led to severe disruption of every stage of biofilm formation (Fox et al., 2015) whereas, Ndt80 is a transcription factor, which was described to exert control over other 'master regulators' involved with biofilm formation (Nobile et al., 2012). This study aimed to investigate the effects exerted by FGE towards *C. albicans* growth and the two important biofilm formation genes as there have been no available data on this.

## MATERIALS AND METHODOLOGY

### Preparation of *Candida albicans* strain and medium

*Candida albicans* ATCC 90028 was grown on 4% Sabouraud dextrose agar (SDA) (Merck, Darmstadt, Germany) for 24 h at 37 °C. The strain was stored in 20% glycerol stock at -20 ºC until further use. Prior to experimental use, *C. albicans* was sub-cultured thrice.

### Fresh garlic extract preparation

Fresh garlic extract (FGE) was prepared using the protocol described by Low et al. (2008) with some modifications. FGE was diluted with sterile, distilled water to achieve the working concentrations of 0.1 mg/mL, 1 mg/mL, 10 mg/mL, and 100 mg/mL for agar well and disc diffusion assays while 50 mg/mL, 100 mg/mL, 200 mg/mL, and 400 mg/mL were prepared for XTT reduction assay. FGE was freshly prepared before each experiment.

### Identification of antifungal properties of fresh garlic extract (FGE)

Overnight culture of *C. albicans* was resuspended in 0.85% of sterile sodium chloride (Merck, Darmstadt, Germany) to obtain an optical density of 0.1 ± 0.02 at 600 nm (Thermo Fisher, U.S.A.), which amount to approximately $1.14 \times 10^6$ CFU/mL. Minimum concentration of FGE required to exert an inhibitory effect was determined by agar well and disc diffusion assays. A volume of 100 µL of *C. albicans* was spread onto the surface of fresh SDA using sterile cotton swabs. Fifty microlitres of each FGE working concentrations at a final concentration of 0.1 mg/mL, 1 mg/mL, 10 mg/mL, and 100 mg/mL was aliquoted into wells that measured 5 mm in diameter for the agar well diffusion assay.

A total of 50 µL of each FGE working concentration at a final concentration of 0.1 mg/mL, 1 mg/mL, 10 mg/mL, and 100 mg/mL was aliquoted onto sterile, blank discs (Sigma, St. Louis, U.S.A.) and left to dry at room temperature. The discs were transferred onto the surface of the spread plates using tweezers, aseptically. A volume of 50 µL of amphotericin B (AMB) (Sigma, St. Louis, U.S.A.) at 0.25 µg/mL (which was the predetermined minimum inhibitory concentration (MIC) for the test *C. albicans*) was prepared in dimethyl sulfoxide (DMSO) (Nacalai Tesque, Kyoto, Japan) and utilised as the positive control for agar well and disc diffusion assays. Sabouraud dextrose broth (SDB) (Merck, Darmstadt, Germany) containing 1% DMSO was utilised as the negative control. Plates for both agar well and disc diffusion assays were incubated at 37 ºC for 24 h. Each assay was conducted in triplicates.

### Determination of antibiofilm properties of FGE

Effects of FGE towards *C. albicans* biofilm was determined using the protocol established by Pierce et al. (2008) with slight modifications. Overnight cultures of *C. albicans* were washed thrice with ice cold 1× PBS and prepared to $1\times 10^6$ CFU/mL in prewarmed RPMI-1640 (Sigma St. Louis, U.S.A.). A volume of 100 µL of culture was aliquoted

into all wells except wells in one column, which were utilised for the controls before the plate was incubated statically at 37 ºC for 24 h. All wells were washed thrice with 200 µL of 1× PBS prior to the addition of 100 µL of respective antifungal concentrations and incubation for 24 h at 37 ºC, statically. Wells were prewashed thrice and 100 µL of prepared XTT/menadione solution were added to each well followed by incubation at 37 ºC for 3 h in the dark. Eighty microlitres of solution from each well was transferred to a fresh plate and read at 490 nm. FGE concentrations tested were ¼×, ½×, 1× and 2× inhibitory concentration (25 mg/mL, 50 mg/mL, 100 mg/mL and 200 mg/mL, respectively) as established by the agar well and disc diffusion assays. AMB at a concentration of 0.25 µg/mL and SDB with 1% DMSO served as the positive and negative controls, respectively. The assay was conducted in pairs for each concentration on three separate occasions. Paired sample t-test was performed between treated and untreated samples whereby, p-value < 0.05 was considered significant.

### Evaluation of FGE effect towards gene expression of *C. albicans* biofilm-associated genes

*Candida albicans* was treated with 5 mg/mL and 10 mg/mL of FGE at 37 $^{O}$C for 24 h to obtain sufficient RNA for gene expression analysis. RNA extraction was performed using RNeasy Mini Kit (Qiagen, Venlo, Netherlands) according to the manufacturer's instructions with some modifications. Sterile glass beads were initially utilised to mechanically disrupt the cell wall prior to the steps provided. Reverse transcription of RNA to cDNA was performed using the One Taq RT-PCR kit (New England Biolabs, Ipswich, Massachusetts, U.S.A.) followed by RT-PCR with Luna Universal One-Step RT-PCR kit (New England Biolabs, Ipswich, Massachusetts, U.S.A.) according to the manufacturer's instructions. Fold change analysis was conducted using the Livak method (Livak and Schmittgen, 2001). Each gene was analysed in triplicate. Primers utilized are as shown below (Table 1).

**Table 1:** Sequences of primers utilized.

| Primers | Sequences | References |
|---|---|---|
| Flo-8 | Forward: TCATAACTACAATTATTGCTGG Reverse: GTTGTTGTTGTTGTTGGCTC | Liu et al., 2015 |
| Ndt80 | Forward: TCCCCAACCAACACCACAAC Reverse: GCATATCGGTCCCCACACAATA | Generated using Primer3 |
| GAPDH (Housekeeping) | Forward: CGGTCCATCCCACAAGGA Reverse: AGTGGAAGATGGGATAATGTTACCA | Nailis et al., 2006 |

## RESULTS

### Antifungal and antibiofilm properties of FGE

Zones of inhibition were noted for AMB and FGE (Table 2). Findings from agar well and disc diffusion assays indicate that only FGE at 100 mg/mL could inhibit the growth of *C. albicans* (Figure 1).

**Table 2:** Diameter of zones of inhibition noted for AMB (0.25 µg/mL) and FGE (100 mg/mL) after 24 h.

| | Zone of Inhibition (mm) | |
|---|---|---|
| | Agar Well Diffusion | Disc Diffusion |
| AMB | 26.0 ± 1.0 | 25.7 ± 1.2 |
| FGE | 35.0 ± 1.0 | 33.3 ± 0.6 |

Diameters are reported in mm as mean ± standard deviation.

AMB and FGE exerted inhibitory effects towards preformed *C. albicans* biofilms. The readings observed (Table 3) indicates that AMB and FGE were able to disrupt *C. albicans* biofilms at ¼× inhibitory concentration.

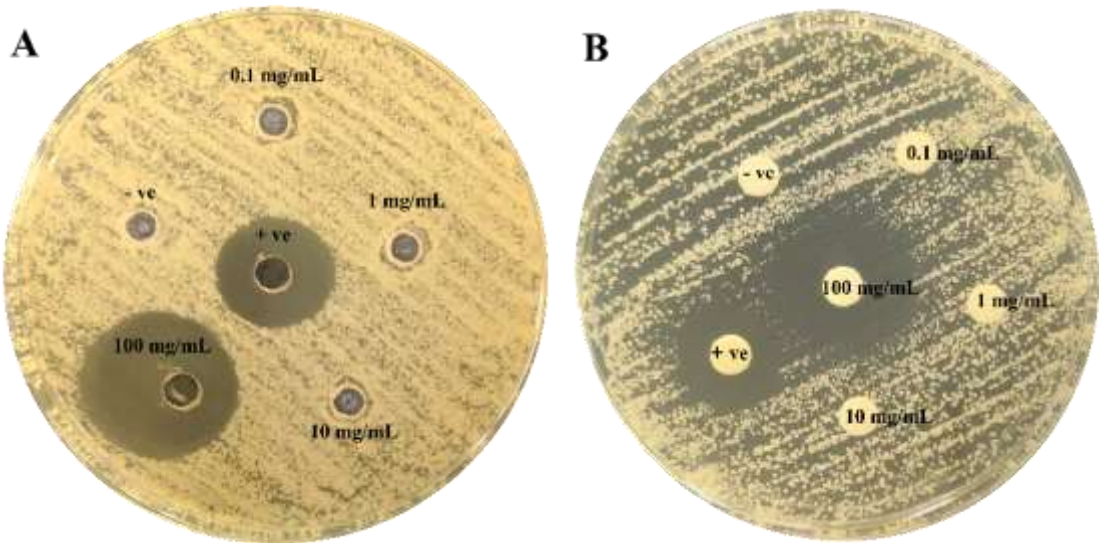


**Figure 1:** Anti-Candida properties exhibited by FGE through (A) agar well and (B) disc diffusion assay after 24 h. Zones of inhibition were visible in the areas surrounding the discs and the wells of 0.25 ug/mL of AMB (+ve control) and 100 mg/mL of FGE, respectively.

**Table 3:** XTT assay of *C. albicans* biofilm when treated with varying concentrations of FGE.

| Concentration of antifungal agents | Mean Absorbance at 490nm ± SD using XTT assay | |
|---|---|---|
| | AMB | FGE |
| Untreated cells | 0.389 ± 0.109 | |
| ¼× inhibitory concentration | 0.053 ± 0.015* | 0.013 ± 0.003* |
| ½× inhibitory concentration | 0.038 ± 0.009* | 0.005 ± 0.002* |
| 1× inhibitory concentration | 0.012 ± 0.005* | 0.002 ± 0.002* |
| 2× inhibitory concentration | 0.002 ± 0.001* | 0.001 ± 0.001* |

FGE; ¼× inhibitory concentration = 25 mg/mL, ½× inhibitory concentration = 50 mg/mL, 1× inhibitory concentration = 100 mg/mL and 2× inhibitory concentration = 200 mg/mL and AMB; ¼× inhibitory concentration = 0.0625 µg/mL, ½× inhibitory concentration = 0.125 µg/mL, 1× inhibitory concentration = 0.25 µg/mL and 2× inhibitory concentration = 0.50 µg/mL. (* indicates p-value ≤ 0.001)

### Effect of FGE on the expression of *C. albicans* biofilm-associated genes

The findings indicate an upregulation of Flo-8 (67.34 and 30.20-folds) and Ndt80 (5.55 and 1.79-folds) when treated with 5 mg/mL and 10 mg/mL of FGE, respectively. Notably, both genes showed higher fold change at 5 mg/mL compared to 10 mg/mL (Figure 2), which indicates that this might be the optimal concentration to observe the effects exerted by FGE towards these genes. Moreover, FGE at the same concentration seems to have more effect towards the expression of Flo-8 compared to Ndt80.

## DISCUSSION

*C. albicans*-associated nosocomial infections are a persistent problem faced by medical practitioners, particularly with the emergence of resistant strains (Savastano et al., 2016). AMB remains one of the effective antifungal drugs available (Mesa-Arango et al., 2012). It functions by binding to ergosterol, a major component in fungal membranes to cause cell death through pore formation as well as through sterol sequestration (Palacios et al., 2011). Prolonged use of this drug has been observed to cause adverse effects to its host (Laniado-Laborín & Cabrales-Vargas, 2009). Meanwhile, garlic is one of many natural compounds that have been studied as an alternative therapeutic due to its promising antimicrobial properties with no sign of severe toxicity at higher doses (Rahman, 2007). Allicin, the major thiosulfinate extracted from garlic has been identified as the key component associated with the antimicrobial properties demonstrated by FGE (Cavallito & Bailey, 1944). It is understood to function by inhibiting the lipid, protein and nucleic acid synthesis of microorganisms by binding to their thiol (-SH) groups (Fujisawa et al., 2009) but the mode of action is still not fully elucidated. Allicin has also been described to inhibit spore germination and hyphal growth (Shadkchan et al., 2004; Khodavandi et al., 2011a).

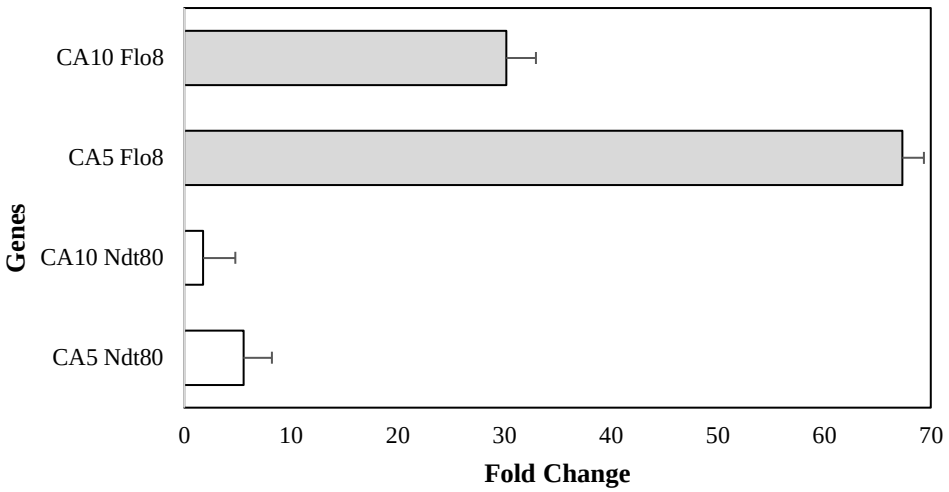


**Figure 2:** Fold change analysis of the expression of Flo-8 and Ndt80 after treatment with FGE (5 mg/mL and 10 mg/mL) derived from RT-PCR.

The present findings indicate that FGE has the ability to inhibit *C. albicans* at a concentration of 100 mg/mL, as previously noted by Low et al. (2008). This finding is also parallel with a research by Lemar et al., (2002) in which FGE was observed to exert fungicidal effects towards *C. albicans* cells by scanning electron microscopy (SEM) and transmission electron microscopy (TEM) at 10 mg/mL. Similarly, the inhibitory effects of FGE were noted in a study to compare the inhibitory effects of FGE, fluconazole and itraconazole whereby, FGE demonstrated an inhibition at 40.7% (w/v) concentration compared to the two commercialised drugs at 15 and 8 µg/mL, respectively (Li et al., 2015).

FGE demonstrated the ability to significantly ($p < 0.001$) inhibit the metabolic activity of *C. albicans* biofilm at ¼× to 2× inhibitory concentration when compared to AMB. The current findings is in support of the observations reported by Shuford et al., (2005) wherein, FGE at 4 mg/mL could effectively reduce metabolic activities at the adherence phase. An attempt to determine the effects of FGE towards the expression of the two biofilm-associated genes, Flo-8 and Ndt80 by *C. albicans* demonstrated that FGE led to the increase of these genes. Flo-8 and Ndt80 are essential for invasive/filamentous switch and hyphal development as well as biofilm formation in response to stress, respectively (Fox et al., 2015). An increase in the expression of both genes in the presence of FGE could be a form of defense mechanism to protect itself from a potentially harmful environment.

Flo-8 plays a major role in every stage of biofilm development as proven through biofilm deficient mutant strains (Fox et al., 2015). Inability of *C. albicans* to form proper biofilms in the presence of FGE may have resulted in the upregulation of Flo-8 as a compensatory response in the present study. Contrary to our initial hypothesis, we postulate that the presence of FGE interfered with the progression of sessile cells to biofilm stage, which led to the overexpression of Flo-8 as the yeast cells attempt to develop into biofilms. Hence, this could indicate that the molecular target of FGE and its active constituents maybe another master regulator upstream of Flo-8 or alternatively, a pathway independent of Flo-8 altogether. Dantas et al., (2015) speculated that oxidative stress could trigger morphogenesis in *C. albicans*, which would also account for the upregulation of Flo-8 in the current study. A study on *Candida tropicalis* demonstrated that an upregulation of hyphae production resulted in an increase of several genes associated with morphological differentiation as well as oxidative response (Jiang et al., 2016). Thus, implying that the increase in Flo-8 might trigger the expression of other genes. Low and co-authors (2008) noted a downregulation of another hyphal development gene, SIR2 at 100 mg/mL of FGE with no significant inhibitory effect observed towards SAP1-4 which code for the virulence factors, secreted aspartyl proteinases. This was further justified by Khodavandi and co-workers (2011b) in their study utilising pure allicin who noted the inhibition of HWP1, a gene involved in the development of hyphal cell wall.

Ndt80 plays a role in the development of resistance under stressful conditions and in hyphal growth (Min et al., 2018). In the case of azole resistance, the overexpression of Ndt80 leads to the increase of the drug efflux pump, CDR1. Presence of FGE might have triggered a stress response in *C. albicans* that led to the increased expression of Ndt80 to pump out potential threats. Aside from that, Ndt80 has been described to repress biofilm formation in *Candida parapsilosis* (Branco et al., 2021). Increase in the expression of Ndt80 in the present study could be a form of antibiofilm activity exerted by FGE. A decrease in expression of Ndt80 at 10 mg/mL demonstrates that FGE might suppress its expression alongside other virulent genes at higher concentration, hence, indicating that FGE has promising antibiofilm activities when administered before biofilms can be established at the appropriate concentrations.

One of the limitations of this study is that the effects of FGE cannot be directly compared to those of AMB as the concentration of the latter was lower at 0.25 µg/mL. Although, previous studies have shown comparable findings between FGE or its derivatives and commercially available antifungals such as fluconazole (Khodavandi et al., 2011a), the present study does not utilise the same drug concentrations. There could be variations in the results obtained as the extraction and preparation method of FGE may vary by batch and extraction method. The

limited range of FGE concentrations tested using RT-PCR may not be sufficient to be conclusive. Moreover, the overall effect of FGE observed in this study is a combined effect of other bioactive compounds such as ajoene, DADS, diallyl trisulfide (DATS) as well as diallyl sulfide (DAS) and should not be solely attributed to allicin.

## CONCLUSION

FGE exhibited antifungal and antibiofilm effects towards *C. albicans*. The present findings indicate that FGE may trigger a response in Flo-8 and Ndt80 as evident by the upregulation of their expression at 1/10 and 1/20× inhibitory concentration. FGE, allicin or its other derivatives exhibit promising antimicrobial properties. However, it would require more in-depth research such as determining the MIC through broth microdilution before it can be utilised as an alternative therapeutic to combat *C. albicans*-associated nosocomial infections.

## AUTHOR CONTRIBUTIONS

Dave Aranka Thomas and Joshua Lee Voon Kai performed the experiments; Choo Sulin supervised the experimental conduct and wrote the manuscript, Harinash Rao supported the research and edited the manuscript. Chong Pei Pei supported, designed, supervised, edited and reviewed the manuscript. All authors read and approved the final manuscript.

## ETHICS APPROVAL

No ethical approval was required as the project did not involve any human nor animal subjects.

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## CONFLICT OF INTEREST

The authors declare no conflicts of interest in this work.

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