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Antioxidative mechanism of *Quercus infectoria* gall aqueous extract against *Candida albicans*

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ABSTRACT

Aims: The effect of *Quercus infectoria* (QI) gall extract on the fungal expression of antioxidant defense enzymes of *Candida albicans* was studied in an effort to unravel its anti-fungal mechanism.
Methodology and results: Minimum inhibitory concentration (MIC), minimum fungicidal concentration (MFC), and time kill assays were conducted to analyse the antifungal activity of the extract against *C. albicans*. Total protein profiles of *C. albicans* were determined by SDS-PAGE and real-time PCR was used to quantify the genes expression level of superoxide dismutase (SOD1), catalase (CAT1) and glutathione peroxidase (GPX3) following treatment with aqueous QI gall extract. The MIC and MFC values of the extract against *C. albicans* were 8 mg/mL and 16 mg/mL respectively. Fungistatic and fungicidal activities of the extract were observed after 24 h at 1× MIC and 2× MIC from the time-kill assay. A lower total protein bands density of extract-treated *C. albicans* was visualized when compared to the untreated cells at concentrations of 1× MIC and 2× MIC. A significant reduction in the expression of GPX3 (p <0.05) was observed following 2 h treatment of extract at 1× MIC and 2× MIC though it did not significantly affect those of SOD1 and CAT1 (p >0.05).
Conclusion, significance and impact of study: In conclusion, QI gall extract exerted an anti-*Candida* activity and it is apparently effective in downregulating the gene expression of GPX3 in *C. albicans*. Of note, the present findings elucidated a preliminary mechanism associated with the organism's survival resilience which represents a key target for the development of anti-*Candida* agents in future.

Keywords: Candida albicans, *Quercus infectoria*, glutathione peroxidase, superoxide dismutase, catalase

INTRODUCTION

Candida albicans is one of the pathogenic yeasts that has the ability to cause various types of diseases in a human. It is a predominant causative agent of candidiasis compared to other *Candida* species (Hawkshead *et al.*, 2016) in which over 80% candidiasis were caused by *C. albicans* (Silva *et al.*, 2012). The clinical spectrum of candidiasis is largely ranging from superficial to invasive infections (Sardi *et al.*, 2013). Oral thrush, vulvovaginal candidiasis and urinary tract infection are some of the most common diseases caused by *C. albicans* in human (Dantas *et al.*, 2015). Besides, it is also associated with invasive infections such as endocarditis and meningitis which are frequently related to high morbidity and mortality. High frequency of candidiasis has created a serious clinical problem worldwide and the infection rate keeps on increasing (Chin *et al.*, 2016). In Malaysia, candidiasis represents about 64.1% among all other fungal infections (Tzar *et al.*, 2013).

Human innate immune system has a sophisticated mechanism to recognize and kill the invading pathogen through an oxidative stress killing mechanism by which neutrophils and macrophages produce reactive oxygen species (ROS) (Weiss *et al.*, 1985). Excessive ROS production causes oxidative damage to the lipid, protein and deoxyribonucleic acid (DNA) of a pathogen which eventually leads to cell death (Slauch, 2011). Despite the mechanisms used by the neutrophils and macrophages to combat the infection, *C. albicans* can survive in the host by expressing the antioxidative defense enzymes (Abegg *et al.*, 2010). These enzymes play a crucial role in shielding the organism from numerous oxidative damages deployed by the host immune system (Hussain *et al.*, 2015).

Infections initiated by *C. albicans* are becoming difficult to treat due to the emergence of strains that are resistant to antibiotics. The genetic alteration of this strain also enhances its virulency which creates another problem for the host to resolve the infections (Gow *et al.*, 2011). The evolution of fungal resistance to the current

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available antibiotics has necessitated the research for more novel antimicrobial compounds.

Many local plants produce a diverse range of bioactive compounds; therefore, they are widely used in traditional medicine. *Quercus infectoria* (QI) gall is one of the herbaceous plants grouped under the family of Fagaceae. In Malaysia, the gall of QI is commonly known as "Manjakani" and it is widely used in traditional medicine. Recently, research in the pharmacological activities of QI gall extract, specifically on their antimicrobial activity has attracted numerous attentions. Previous study has documented that QI gall possesses numerous medicinal properties such as antimicrobial (Basri *et al.*, 2005), antidiabetic, anti-inflammatory as well as antioxidant properties (Aivazi and Vijayan, 2009).

Previous research also has reported that QI gall extract has an antifungal activity against *C. albicans* (Baharuddin *et al.*, 2015), however its mechanism in inhibiting or killing the microorganism is still unclear. Besides, the effect of the extract on gene expression of the antioxidative defense enzymes in *C. albicans* that encode superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX) is still yet to be explored. These enzymes which are fundamental protectors against all forms of stress play a crucial role in shielding the organism from numerous oxidative stresses deployed by the phagocytes to hinder the killing mechanism by a host, allowing pathogens to survive and finally induce disseminated infection. Therefore, the aim of this research was to determine the effect of QI gall extract on the expression of antioxidative defense enzymes in *C. albicans*. The present study demonstrated QI gall extract able to downregulate the antioxidative enzyme gene expression in *C. albicans*. Therefore, this elucidated mechanism associated with the organism's survival resilience represents a key target for the development of anti-*Candida* agents in future.

MATERIALS AND METHODS

Plant material and preparation of QI gall extract

Quercus infectoria (QI) galls were purchased from the local herbal shop in Kota Bharu, Kelantan and identified based on its physical appearances in which the shape was globose or rounded with rough surface, associated with 1.0 to 1.5 cm external diameter, greyish-brown to brownish-black in color and had a slight odor (Vanga *et al.,* 2017). The galls were then washed with distilled water and left to dry at room temperature. Prior to extraction, the galls were crushed and ground to powder. *Quercus infectoria* gall aqueous extraction was performed according to the method described by Baharuddin *et al.* (2015). Aqueous extract of QI gall was prepared by immersing 100 g of gall powder into a sterile bottle containing 500 mL of sterile distilled water and placed in a 50 °C water bath for 72 h. Then, the crude extract was filtered using Whatman filter paper No. 1 and concentrated under vacuum using a rotary evaporator (Heidolph, Germany). The resulting pellet was freeze-dried at –50 °C under vacuum until a fine crystal-like crude extract was produced. The crude extract was stored in an airtight jar at 4 °C until future use.

Fungal strains and preparation of a standardized inoculum

Candida albicans American type culture collection (ATCC) 10231TM (Virginia, USA) was used in this study. The organism was maintained on Sabouraud dextrose agar (SDA) at 35 °C for 24 h. For all assays, the standardized inoculum of *C. albicans* was prepared at 1.5 × 10^6 CFU/mL by adjusting the suspension into 0.5 McFarland standard.

Determination of minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC)

The MIC of QI gall aqueous extract against *C. albicans* was determined by using broth microdilution susceptibility test method as described by CLSI (2012)**.** A 96-microtiter plate was used, and the assay was performed in triplicates. One hundred µL of stock extract at 64 mg/mL was serially diluted in 100 µL sterile distilled water to produce 10 different extract concentrations ranging between 16 mg/mL and 0.03 mg/mL. Then, 100 µL of the prepared inoculum were pipetted into every well containing extract (final concentration of 7.5 × 10^5 CFU/mL). For the positive control, 100 µL of the inoculum was used alone, while extract without inoculum served as negative control. The plate was then incubated at 35 °C for 24 h. The MIC value was defined as the lowest concentration of extract in the well that showed no turbidity (clear broth) by visual inspection (Bajpai *et al.*, 2012). Determination of MFC was done by subculturing the broth with no turbidity or visible growth onto SDA plates (Pfaller *et al.*, 2004). The plates were incubated at 35 °C for 24 h. The MFC was defined as the lowest concentration of extract that grew less than 8 colonies (99.9% cell killing) on the plates.

Time-kill kinetic assay

Time-kill assay was conducted based on the method described by Klepser *et al.* (1998). The standardized inoculum was treated with QI gall extract at concentrations of 0.5× MIC, 1× MIC, 2× MIC and 4× MIC. Then, 1 mL of *C. albicans* suspension was added into 9 mL of extract containing RPMI 1640 medium. The inoculum treated with amphotericin B (100 µg/mL) served as positive control, while inoculum alone was used as negative control. All tubes were then incubated at 35 °C. At predetermined time points (0, 2, 4, 6, and 24 h), 0.1 mL aliquot was removed and subsequently diluted into 3 times dilution before plated onto the SDA. The colony forming unit (CFU) of the organisms was determined. The procedure was performed in triplicate (three independent experiments) and a graph of the $\log_{10}$ CFU/mL against time was plotted.

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Analysis of total protein profile by SDS-PAGE

The fungal inoculum was treated with QI gall extract at the fungistatic and fungicidal concentrations for 2 h at 35 °C. The untreated inoculum served as a control. Extraction of protein was performed using Y-PER™ yeast protein extraction reagent (Thermo scientific, South Africa). The concentration of the harvested protein was determined by using NanoDrop™ 1000 spectrophotometer (Thermo scientific, South Africa). The extracted proteins (18.6 mg/mL) were subjected to SDS-PAGE by using 4% (w/v) stacking gel and 12.5% (w/v) separating gel at condition of 100 V for 10 min and 150 V for 1 h, respectively. The gel was then stained with Coomassie blue (R-250) staining solution [0.3% (w/v) coomassie brilliant blue R-250 in 45% (v/v) methanol, 40% (v/v) acetic acid] for 30 min and de-stained overnight on a horizontal shaker (Thomas Scientific, USA) at 80 rpm. The protein bands were analysed by PageRuler™ Plus Prestained Protein Ladder, 10 to 250 kDa (Thermo Scientific, South Africa) as a molecular weight marker for SDS-PAGE.

Analysis of gene expression by Real-Time PCR (RT-qPCR)

The expression analysis of superoxide dismutase (SOD1), catalase (CAT1) and glutathione peroxidase (GPX3) genes from the extract-treated *C. albicans* was performed according to the method which was previously described by Hussain *et al.* (2015) and Khan *et al.* (2015). *Candida albicans* inoculum was treated with the extract at fungistatic and fungicidal concentrations for 2 h. The untreated inoculum served as a negative control whereby the extract was replaced with distilled water. Total RNA from each sample were isolated using Qiagen RNeasy Mini Kit (Qiagen, Germany) according to the manufacturer's instruction. The RNA concentration was determined using NanoDrop ND-1000 spectrophotometer. cDNA synthesis was performed using ReverAid First Strand cDNA synthesis Kit (Thermo Scientific, South Africa) according to the manufacturer's protocol.

Primers for target genes were designed by using Primer3Plus software to identify a unique region that was suitable for amplification. For endogenous control, actin (ACT1) was used as a housekeeping gene. Real time quantification was performed by using PowerUp™ SYBR™ Green Master Mix (Applied Biosystem. USA) according to the manufacturer's protocol with 20 µL of the final volume. Instrument from Applied Biosystem 7500 Real-Time PCR System was used in this study. The primer sequences and annealing temperature used in this study are shown in Table 1. The experiment was performed in triplicate. Ct values of each target gene were used to calculate the gene expression ratio using $2^{-\Delta\Delta CT}$ (Livak method). Relative quantification was used to find the relative expression of the target genes in the untreated and treated *C. albicans* with reference to the housekeeping gene, ACT1, for normalization.

Table 1: List of primers for respective genes used in this study.

Gene	Primer sequence (5'→3')		Annealing temperature (°C)
	Forward primer	Reverse primer	
SOD1	GTC GCT GTT GTC AGA GGT GA	GGA CCA GCA GAA GTA CAA CCA	60
CAT1	TTG GTC AAC ACG GTC CAT TG	ACC ATA AGC ACC GGA ACC TT	60
GPX3	CGC AAT TTT GGT GTC AGT TTC C	AAA ACC CAC TTC ACC AGG CT	60
ACT1	GTT TTG GAT TCT GGT GAT GGT GTT	TCA AGT CTC TAC CAG CCA AAT CAA	60

Statistical analysis

Data obtained were statistically analysed using IBM SPSS statistic 22. For determination of mean differences in antioxidative defense enzyme gene expression, the normality and homogeneity of variance were initially tested through the Shapiro-Wilk test. Then, one-way ANOVA with Tukey's HSD post hoc test was performed for statistical comparison between groups. The value of $p < 0.05$ was considered as statistically significant.

RESULTS

Antifungal activity

The MIC value of the aqueous QI gall extract against *C. albicans* was 8 mg/mL, while the MFC value was 16 mg/mL. Results of the time-kill kinetic study are presented in Figure 1. After 2 h of treatment with extract at 0.5× MIC (4 mg/mL), the growth of *C. albicans* had declined initially, then it resumed but slower than the untreated cell growth after 4 to 24 h. Meanwhile, continuous growth inhibition was observed when the cells were treated with extract at 1× MIC (8 mg/mL) compared to the growth control. After 6 h of extract treatment with 2× MIC (16 mg/mL) and 4× MIC (32 mg/mL), the viable cell count showed a decrease of 1 $\log_{10}$ CFU/mL and more than 2 $\log_{10}$ CFU/mL respectively. The fungicidal effect of the extract was seen at concentrations of 16 mg/mL and 32 mg/mL at 24 h of incubation (Figure 1). Therefore, the minimum fungistatic and fungicidal concentrations of extract was determined at 8 mg/mL and 16 mg/mL respectively.

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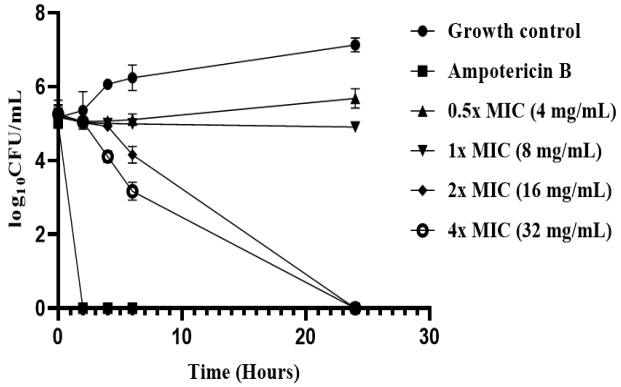

Figure 1: Time-kill kinetic assay of *C. albicans* treated with different concentrations of QI gall extract. The experiment was performed in triplicate.

Analysis of protein profile

Protein band patterns in both untreated and extract-treated *C. albicans* were clearly separated and visualized in Figure 2. Overall, the protein band intensity of *C. albicans* treated with extract at 1× MIC (8 mg/mL) and 2× MIC (16 mg/mL) had reduced compared to the untreated cells when analysed qualitatively. The antioxidative defense proteins were estimated at 16.15 kDa for SOD1 (Jones *et al.*, 2004), 54.88 kDa for CAT1 (Wysong *et al.*, 1998) and 19.85 kDa for GPX3 (Cuomo *et al.*, 2013).

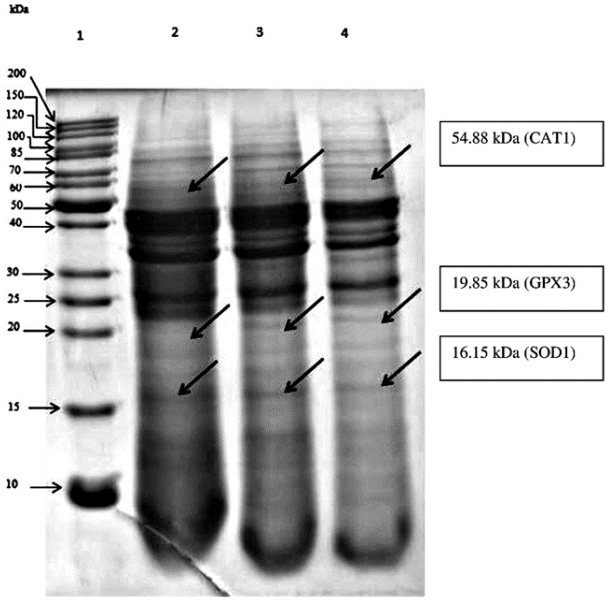

Figure 2: Zymography of cytoplasmic proteins analysed by SDS-PAGE following 2 h treatment of *C. albicans* with QI gall extract at 35 °C. Lane 1: protein ladder; Lane 2: untreated cells; Lane 3: extract-treated cells at 8 mg/mL; Lane 4: extract-treated cells at 16 mg/mL.

Analysis of gene expression

The effect of QI gall extract on the expression of SOD1, CAT1 and GPX3 genes, encoding superoxide dismutase, catalase and glutathione peroxidase respectively in *C. albicans* was investigated. The relative expression of the target genes in the treated and untreated samples was determined in reference to ACT1 as the internal control. The amplification efficiencies for SOD1, CAT1, GPX3 and ACT1 were 108%, 100%, 98% and 99% respectively (Supplementary Figure 1).

Figure 3 shows the relative expression of SOD1 in the treated and untreated cells. Based on the result obtained, there was no significant changes (p= 0.72) indicated in the relative expression of SOD1 after being treated with extract at 1× MIC (8 mg/mL). Meanwhile, treatment with extract at 2× MIC (16 mg/mL) for 2 h showed a slight decrease in the relative expression of SOD1 to 0.64-fold compared to untreated cells which was not statistically significant (p= 0.14) to indicate the downregulation of SOD1 expression.

Treatment with both 1× MIC (8 mg/mL) and 2× MIC (16 mg/mL) showed no significant changes in the relative expression of CAT1 after 2 h compared to the untreated cells (Figure 3). On the other hand, the relative expression of GPX3 was significantly reduced to 0.54-fold (p = 0.04) and 0.4-fold (p= 0.01) with 1× MIC and 2× MIC extract treatment respectively as compared to the untreated cells.

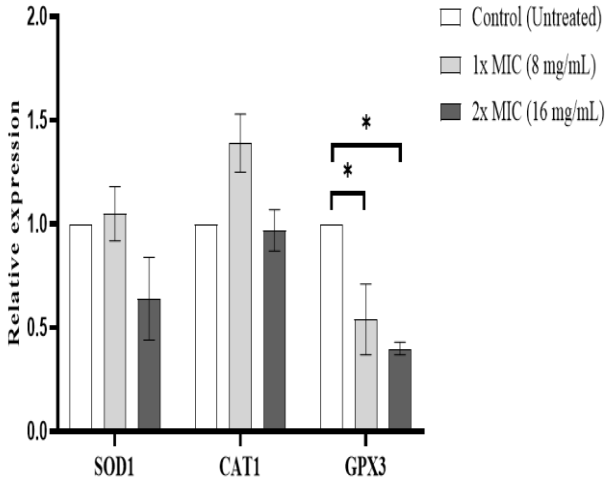

Figure 3: Relative expression of SOD1, CAT1 and GPX3 genes expression in QI gall extract-treated *C. albicans*. Asterisk (*) indicates a statistically significant difference (p <0.05) between the test groups and negative control group (untreated). Comparison of the gene expression level between groups (untreated cells, treated cells with 1×MIC and 2×MIC) was determined by one-way ANOVA, followed by Tukey post hoc test.

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DISCUSSION

The MIC and MFC values of QI gall extract against *C. albicans* in this study are slightly higher than the results from the previous study by Baharuddin *et al.* (2015) which reported the MIC value of 1 mg/mL and MFC of 4 mg/mL This possibly occurred due to some variations in the technical aspects which include environment conditions of the experiments and operator skills. The variation of the extract biological activities can also be due to different extraction method applied and its functional properties are largely contributed by the amount of bioactive compound in the extract (Quispe-Condori *et al.*, 2008). Different source of plant used also might have contributed to this difference. According to Atanasov *et al.* (2015), chemical constituents contained inside the herbs are varied depending on the growing location and the environmental conditions. Factors such as ground and the soil where the plant was grown may contribute to this variation.

Based on time-kill kinetic study, the inhibitory effect of the extract can be observed even at the lowest concentration used (4 mg/mL) and it is apparently fungistatic at the concentration of 8 mg/mL. This could be due to the interference on the protein production, cellular metabolism and DNA replication of the treated organism (Leela and Satirapipathkul, 2011). The fungistatic effect of antimicrobial helps the host immune defense to destroy the invading pathogen (Pfaller *et al.*, 2004). Meanwhile, the fungicidal activity of an antimicrobial agent is defined as greater than 3 $\log_{10}$ -fold decrease in CFUs (surviving bacteria), which is equivalent to 99.9% killing of the inoculum at a point of time compared to at 0 hr (Klepser *et al.*, 1997). This study showed that the minimum extract concentration to kill 99.9% of *C. albicans* following 24 h of treatment was 16 mg/mL. The fungicidal activity of extract was found to be less potent compared to the antifungal reference drug (amphotericin B at 100 µg/mL) in which it completely killed the organisms after 2 h of treatment.

The killing activity of QI gall extract against *C. albicans* was found to be concentration dependent. Therefore, fungistatic and fungicidal concentrations of the extract were subsequently chosen in SDS-PAGE and RT-qPCR analysis in order to further analyse its effect on organism's protein profile and gene expression. This study showed that the protein production of *C. albicans* was affected after being exposed to the extract at fungistatic and fungicidal concentrations for 2 h (Figure 2). The growth inhibition is possibly due to the loss of protein function, leading to interference of normal cellular process. The suppression of the organism's protein synthesis could be one of the mechanisms of the antimicrobial killing effect. However, a more specific test like Western blot or flowcytometry analysis is necessary to confirm the expression of specific proteins.

RT-qPCR is another method of choice in the detection of mRNA as it allows a sensitive and specific quantitation of nucleic acids (Ponchel *et al.*, 2003). Relative quantification method was used in this study to describe the relative expression of the target genes between untreated and treated *C. albicans*. Target genes selected for this study were SOD1, CAT1 and GPX3 to encode antioxidative defense enzymes present in *C. albicans* as these enzymes are among important virulence factors in the organism for establishment of systemic model of infections (Abegg *et al.*, 2010). In a human body, the immune system plays a pivotal role in surveillance as well as elimination of any fungal invasion by releasing a set of chemicals which is known as reactive oxygen species (ROS). The chemical released is extremely toxic which can cause significant damage to the pathogen (González-Párraga *et al.*, 2003). However, the presence of these enzymes protects *C. albicans* from the killing effect of ROS by catalysing the conversion ROS into a less harmful chemical thus ensuring its survival (Dantas *et al.*, 2015). Survivability of pathogen inside the body has a potential to create more dangerous infection.

SOD1 plays a crucial role in protecting pathogen from oxidative stress induced by immune cells. This enzyme catalysed the conversion of superoxide anions released by macrophage into less harmful molecule of hydrogen peroxide (H_2O_2) and oxygen (O_2). SODs are required for *C. albicans* to resist the macrophage-mediated killing induced following phagocytosis (Hwang *et al.*, 2017). CAT1 is also one of the crucial antioxidative defense enzymes in *C. albicans*. This enzyme is consistently ready to neutralize ROS encountered extracellularly. CAT1 spontaneously converts hydrogen peroxide (H_2O_2) into oxygen (O_2) and water (H_2O). GPX3 is an essential enzyme in detoxification of hydrogen peroxide to overcome the harmful effect to pathogen (Miramón *et al.*, 2014).

The amplification efficiencies of the target genes in this study were 108% for SOD1, 100% for CAT1, 98% for GPX3 and 99% for ACT1. The ideal level of amplification efficiency must be in between 95 % to 110% to ensure the results obtained are reliable (Bustin *et al.*, 2004). Therefore, the quantification of the mRNA state levels present in the untreated and treated samples in this study are reliable to be quantified and analysed. Based on the results obtained, a significant downregulation of GPX3 gene (p<0.05) which encoded for GPX enzyme in *C. albicans* suggested that suppression of GPX3 gene expression could be one of the fungicidal mechanisms of QI gall extract against *C. albicans*.

Meanwhile, the fungistatic and fungicidal concentrations of the extract did not significantly reduce the gene expression of SOD1 and CAT1. However, a reduction trend in the relative gene expression SOD1 and CAT1 was observed when the cells were treated with extract at fungicidal concentration (16 mg/mL) compared to the untreated cells (Figure 3). It is possible that the level of active compound present in the extract at the tested concentration was not sufficiently high to affect the expression of the target genes. According to the Siqueira *et al.* (2012), the presence of sufficient phytochemical compounds is essential for an optimum effect of plant extract. In addition, treatment time is also one of plausible factors that may affect the study results. As such, a significant reduction in the SOD1 and CAT1 genes

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expression might be observed if the incubation time is increased.

In a previous study, aqueous QI gall extract had significantly downregulated a catalase gene expression in the methicillin resistant *Staphylococcus aureus* (Khairon *et al.*, 2016). Besides that, its protein expression was also reduced. These findings suggest the potential of QI gall extract to interrupt the antioxidative defense system in other pathogens. Meanwhile, another study conducted by Khan *et al.* (2015) found that treatment with monoterpene phenol compound had compromised the antioxidative defense system by affecting the regulatory functions of several antioxidative defense enzymes (SOD, catalase, GPX, glutathione S-transferase and glutathione reductase) in *C. albicans*. Toxic oxidizing effects of this compound on the antioxidative defense system had consequently weakened its function in protecting the organism against the damaging effect of ROS generated by the immune cells.

Based on Baharuddin *et al.* (2015), phenol is among of major phytochemical compounds contained in the aqueous QI gall extract. The inhibition of the GPX3 in the present study might be due to the action of phenol presents in the extract. According to Ultee *et al.* (2002), hydroxyl groups and alpha, beta-double bonds in the phenolic compound play an important role in the antimicrobial activity. Thus, further investigations involving isolation and identification of the active phytochemical components responsible for the observed effects are required and we believe that it may open the door for further studies on its use in therapy of fungal infections.

CONCLUSION

In conclusion, our data suggests that QI gall extract could exert its immunomodulatory activity via modulation of the fungal antioxidative defense enzyme expressions and may have the potential in inducing the oxidative killing effect on *C. albicans*. Of note, the present findings elucidated a preliminary mechanism associated with the organism's survival resilience which represents a key target for the development of anti-*Candida* agents in future.

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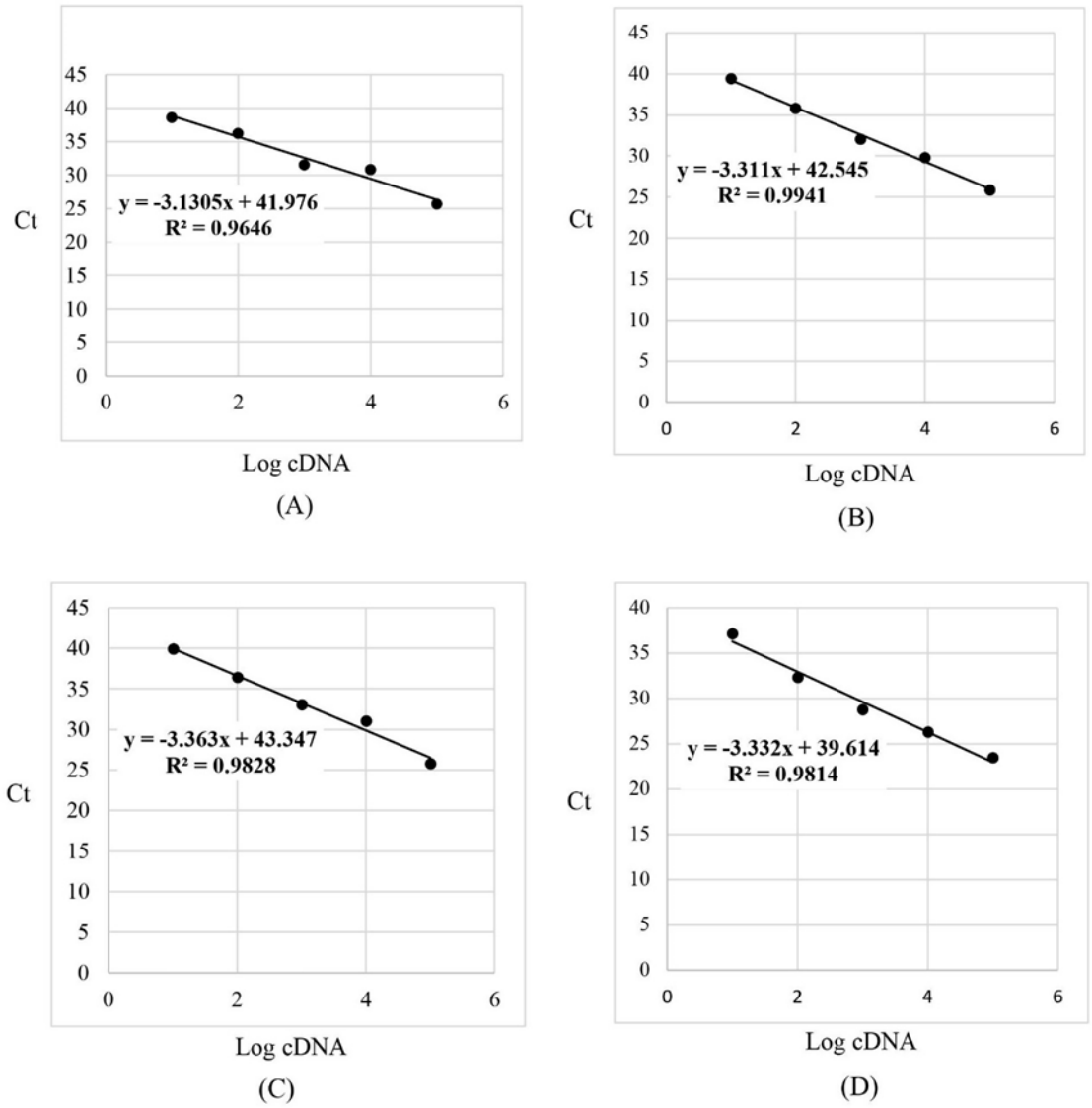
SUPPLEMENTARY INFORMATION



Supplementary Figure 1: Standard curve for target genes. A, SOD1; B, CAT1; C, GPX3; D, ACT1. Amplification efficiencies for SOD1, CAT1, GPX3 and ACT1 were 108%, 100%, 98% and 99% respectively.

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