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# Investigation of Fluconazole Susceptibility to *Candida Albicans* by MALDI-TOF MS and Real-Time PCR for *CDR1, CDR2, MDR1* and *ERG11*

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# Abstract

**Background** *C. albicans* is the most important yeast that caused the infection in humans; the trend of resistance to fluconazole (FLC) was also increased, while the FLC susceptibility by conventional method takes time causing the treatment failure. To investigate FLC susceptibility to *C. albicans* using MALDI-TOF MS and Real-time PCR for *CDR1, CDR2, MDR1* and *ERG11*, overall, 32 *C. albicans* strains included 4 reference strains (3 FLC susceptible (S) and 1 FLC resistant (R), 1 spontaneous mutant strain (FLC susceptible-dose dependent, SDD) and 27 clinical strains obtained from 2 Thai University Hospitals were performed FLC susceptibility testing by Sensititre YeastOne and broth microdilution method, FLC resistant expression mechanism by Real-time PCR and the major peak determination by MALDI-TOF MS.

**Results** The change of *CDR1* and *CDR2* mRNA expression were only significantly observed in SDD and R strains. Using MALDI-TOF MS, the change of mass spectral intensity at range 3376-3382 m/z (major peak) was significantly related to FLC susceptibility as SDD (decreased at 4 μg/ml and increase at 8 μg/ml), S (all increased), and R (all slightly decreased or no change) after incubation for 6 h. All 27 clinical strains showed FLC MIC susceptible (MIC range 0.25-2 μg/ml), no change in *CDR1* and *CDR2* expression and S major peak type. The FLC resistance *C. albicans* with *CDR1* and *CDR2* expression may possibly effect the change of mass spectral intensity at range 3376-3382 m/z.

**Conclusions** The MALDI-TOF MS may be used to simultaneously classify and predict FLC resistant *C. albicans* strains associated with *CDR1* and *CDR2* expression. Further studies are essential to clarify the methodology and improve the reliability of this assay for routine diagnosis.


# Background

Currently, the situation of fungal diseases especially candidemia in hospital is increasing the risk of morbidity and mortality [1]. *Candida albicans* is the most common reported species followed by *C. glabrata*, *C. tropicalis, C. parapsilosis* and *C. krusei* (*Pichia kudriavzevii*), respectively [2]. Fluconazole (FLC), a triazole antifungal is the most widely used for this infection. Fluconazole has a fungistatic effect by inhibiting cytochrome P450 enzyme lanosterol demethylase (14α-demethylase), encoded by *ERG11*, in the ergosterol biosynthesis pathway. Many *C. albicans* clinical isolates overexpress *ERG11*, the gene encoding the target of the azoles. However, in many cases, the level of overexpression is minimal or else observed in combination with other resistance mutations, making it difficult to assess the direct impact of such overexpression on the resistant phenotype. In addition to *ERG11* overexpression, two main classes of efflux proteins included ATP-binding cassette (ABC) transporters encoded by *CDR1* and *CDR2* and Major facilitator superfamily (MFS) encoded by *MDR1* can mediate resistance to FLC by reducing the effective intracellular drug concentration [3, 4]. The azole resistance associated with *ERG11*, *MDR* or *CDR* overexpression has been reported in many cases [5, 6]. For the time being, broth microdilution (BMD) method, a gold standard of antifungal susceptibility testing (AFST) of *Candida* spp. was standardized and determined MIC level of antifungal agents by Clinical and Laboratory Standards Institute (CLSI) and European Committee on Antibiotic Susceptibility Testing (EUCAST). Notwithstanding, this method is time-

consuming and tedious steps. The matrix assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) is generally used for microbial identification by mass spectrum analysis compared with the databases [1]. Several studies were adapted the MALDI-TOF MS for testing susceptibility to bacteria and fungi AFST because of simple, high-throughput, waste reducing, rapid and low expense method [7–9]. The aim of this study was to investigate FLC susceptibility to *C. albicans* using MALDI-TOF MS and Real-time PCR for *CDR1, CDR2, MDR1* and *ERG11*.

# Methods

### Yeast Samples used in this study

Clinical samples of *C. albicans* in this study were 15 strains collected from Microbiology Laboratory Unit, Department of Central Laboratory and Blood Bank, Faculty of Medicine, Vajira Hospital, Navamindradhiraj University, Bangkok, Thailand since April 2015 to December 2017, and 12 strains obtained from positive blood culture in Microbiology Laboratory, Thammasat University Hospital from January 2013 to April 2016. Four ATCC standard strains were used as the control strains including 2 susceptible strains (*C. albicans* MYA2876 and *C. albicans* ATCC90028), 1 strain for spontaneous mutation in SDD MIC level (*C. albicans* MYA4440), and 1 resistant strain (*C. albicans* ATCC96901). *C. parapsilosis* ATCC22019 and *C. krusei* ATCC6258 were used as quality control strains for susceptibility testing.

### Sensititre YeastOne Antifungal Susceptibility testing

Twelve strains collected from Thammasat Hospital were subcultured on Sabouraud Dextrose Agar (SDA) and incubated at 35°C overnight. They were performed using Sensititre YeastOne (SYO) according to the manufacturer's instructions (Thermo Fisher Scientific, Cleveland, OH, USA) for measuring MIC of 9 drugs included fluconazole, anidulafungin, amphotericin B, micafungin, caspofungin, 5-Flucytosine, posaconazole, voriconazole and itraconazole. The SYO plate was incubated at 35°C overnight and MIC endpoints were read after 24 h of incubation by the color changed from blue (negative, indicating no growth) to magenta (positive, indicating growth) [18].

### CLSI Broth microdilution method (CLSI BMD)

Reference BMD testing was performed exactly as outlined in CLSI document M27-4$^{th}$ ed [19], with a final inoculum concentration of 0.5 x $10^3$ to 2.5 x $10^3$ cells/ml and RPMI 1640 medium with 0.2% glucose , and incubation at 35°C. MIC values were visually observed for the presence or absence of growth after 24 h (*Candida* and other yeast) and 72 h (*C. neoformans)* of as the lowest concentration of drug that produced a prominent decrease in turbidity (ca. 50% growth reduction) relative to that of the drug-free control.

### Susceptibility breakpoints

The MIC (μg/ml) interpretations follow the CLSI breakpoints for fluconazole (≤2 μg/ml; Susceptible, 4 μg/ml; Susceptible-dose dependent, ≥8 μg/ml, Resistant). Interpretation of susceptibility was performed by applying the CBPs defined by CLSI [20]. In the CBPs absence, isolates were defined as having a wild-type or a non-wild-type drug susceptibility phenotype including amphotericin, 5-fucytosine, itraconazole and posaconazole according to the epidemiological cutoff values determined by SYO [18, 21].

### Selection of spontaneous mutants

*C. albicans* MYA4440 isolates (MIC = 0.5 μg/ml) and G950 isolates (MIC = 2 μg/ml) were subcultured and incubated at 35°C overnight. The individual colony from each strain was used to start cultures in RPMI and grown overnight. One hundred microliters of overnight cultures ($5x10^8$ to $1x10^9$ CFU/ml) were streaked onto SDA plates with (1, 1.5, 2, and 4 μg/ml) or without fluconazole and incubated at 35°C for 2 days. MIC was checked and resubcultured onto a fresh-passage until the MIC was 4 μg/ml. The spontaneous mutation frequency rate was calculated as the ratio of viable colonies growing on drug-containing plates over the starting inoculum. Mutant resistance phenotypes were confirmed by subculturing on SDA plates containing an amount of drug equivalent to that used for initial selection. Mutant strains were selected and evaluated by MIC and qRT-PCR.

### RNA extraction, cDNA synthesis and Quantitative real-time PCR (qRT-PCR)

Each *C. albicans* isolate was suspended in 5 ml of yeast nitrogen base (YNB) medium supplemented with 50 mM glucose and incubated at 37°C in a shaker at 75 rpm overnight to prepare each of the starters. After that, 500 μl of the starter was transferred into a flask of 50 ml containing fresh medium and incubated at 37°C in a shaker at 75 rpm until $OD_{600}$ = 1.0. Total RNA was extracted from the growth medium (at 0 h without fluconazole and after 24 h with 8 μg/ml of fluconazole addition) at mid-exponential (log) phase using the preparation of yeast RNA by extraction with hot acidic phenol. The concentration of RNA was measured using a NanoDrop 2000C spectrophotometer (Thermo Scientific). The RNA purity and integrity was evaluated by the ratio of absorbance 260 and 280 nm. Run gel electrophoresis will be performed to verify that the RNA was intact. Total RNA was treated with TURBO DNA-*free*™ Kit (Invitrogen) according to the manufacturer's instructions (RNA sample >200 μg nucleic acid per ml). cDNA was synthesized by reverse transcription from 1 μg of total RNA using the iScript™ Reverse Transcription Supermix for RT-qPCR (Biorad) according to the manufacturer's instructions. The reaction protocols for cDNA synthesis were composed of priming step at 25°C for 5 min, reverse transcription step at 46°C for 20 min, and RT inactivation step at 95°C for 1 min. The mRNA expression level was measured using quantitative real-time RT-PCR (qRT-PCR) following Watamoto's protocol [22]. The sequences primers (macrogen) of the genes *CDR1, CDR2, MDR1, ERG11* [22], and *PMA1* [23] in this study are listed in Table 3. qRT-PCR was performed in duplicate using iTaq™ Universal SYBR® Green Supermix (Biorad). Ten microlitres of PCR mix (5 μl iTaq™ Universal SYBR® Green Supermix, 1 μl primer mix, 0.5 μl cDNA and 3.5 μl DEPC water) was used for each gene and qRT-PCR was performed using the following cycling conditions: 95°C for 5 min, followed by 40 cycles of 95°C for 15 s and 60°C for 30 s. Fluorescence intensities were quantified using Bio-Rad® CFX96™ (Biorad). The relative quantities of the

target genes (*CDR1, CDR2, MDR1* and *ERG11*) were normalized against *PMA1* housekeeping gene expression (plasma membrane ATPase pump). The analyses of genes expressions were performed using the comparative $2^{(-\Delta\Delta CT)}$ method of relative quantification. The mRNA expression level of each target gene was analyzed. All results were presented by mean ± SD. Statistical analyses were performed using GraphPad Prism 5.0 software. Results were compared using one way ANOVA followed by Bonferroni's post test; the results were considered statistically significant when the p value was < 0.0001.

### Detection of mass spectral analysis using MALDI-TOF MS

Fresh-isolated colonies on SDA were cultured in RPMI 1640 medium, adjusted turbidity with 0.5 McFarland Standard, and incubated at 37°C overnight. Two milliliter (ml) of suspension was added into RPMI 1640 medium with different fluconazole concentrations: 4 and 8 μg/ml, and without fluconazole as a negative control. The suspension was incubated at 37°C and harvested at 6 h intervals and centrifuged and separated the pellets and the supernatant. The pellets were extracted with the modified of formic acid extraction following Bruker Daltonics recommended protocol for analysis of *C. albicans* protein spectrum. Briefly, 300 μl of HPLC grade water and 700 μl of ethanol were added, mixed with vortex for 1 min, and centrifuged 13,000 rpm for 3 min. The solution was discarded after centrifugation. Then, the pellets were dried; 70% formic acid was added until covered the pellet, added the silica bead into the solution, and mixed with vortex for 2 min. Acetonitrile was added the equal volume of 70% formic acid (ratio 1:1). The mixer was centrifuged at 13,000 rpm for 3 min. One microliter of supernatant was spotted on the MALDI target plate and dried at room temperature (RT). One microliter of 2.5% TFA of α-Cyano-4-hydroxycinnamic acid (HCCA) matrix was covered and dried at RT again. The sample was analyzed by MALDI-TOF MS (Bruker Daltonics, Bremen, Germany). The supernatant use for fluconazole spectrum analysis was dropped onto plate and dried, covered with 0.1% TFA of HCCA matrix, dried at RT, and analyzed by Bruker Autoflex Speed MALDI-TOF MS. The bacterial test standard (BTS), the ribosomal proteins of *Escherichia coli* strain DH5alpha, was used as a calibrator. The spectrum was measured by flexControl 3.3 software [13, 24] using linear mode (2,000 – 20,000 m/z) for *C. albicans* spectrum and using reflector mode (0 – 3,500 m/z) for fluconazole spectrum. Finally, the data was processed and compared using flexAnalysis.

## Results

### MIC level of control strains, clinical strains and spontaneous mutant strains

Of the FLC susceptibility testing with 4 reference strains, *C. albicans* MYA2876, *C. albicans* ATCC90028 and *C. albicans* MYA4440 were susceptible (S) to FLC at MIC level of 0.25, 0.25 and 0.5 μg/ml, respectively, whereas *C. albicans* ATCC96901 was resistant to FLC at MIC level of ≥64 μg/ml. With spontaneous mutation process up to 10 consecutive serial passages, *C. albicans* MYA4440 mutant (ΔMYA4440) were susceptible-dose dependent to FLC (MIC = 4 μg/ml), while G950 strain was not change in MIC level. All 27 clinical strains of *C. albicans* were susceptible to FLC at MIC range 0.5-2 μg/ml (Table 1). Twelve strains collected from Thammasat University Hospital using Sensititre YeastOne

(SYO) were susceptible to fluconazole, voriconazole, anidulafungin, caspofungin and micafungin when applying CLSI CBPs. When applying the epidemiological cutoff values (ECVs), all the isolates had wild type phenotype drug susceptibility to amphotericin, 5-flucytosine, posaconazole and itraconazole (Table 2).

### *CDR* efflux pump overexpression

Figure 1a and 1b indicated *CDR* efflux pump overexpression in *C. albicans* strains, the SDD (ΔMYA4440) and R (ATCC96901) strains significantly overexpressed both *CDR1*and *CDR2* (p-value < 0.0001) at higher level than S strains. Compared to *C. albicans* MYA4440 (wild type) strain (MIC 0.5 μg/ml), its mutant strain (ΔMYA4440) at MIC level of 4 μg/ml expressed *CDR1* and *CDR2* at higher level. After incubation with 8 μg/ml of FLC for 24 h, the SDD strain (ΔMYA4440) and R strain (ATCC96901) significantly overexpressed *CDR1* (p-value < 0.0001) but significantly repressed *CDR2* (p-value < 0.0001), while S strains (MYA2876, ATCC90028 and MYA4440) and all clinical S strains were nonsignificantly expressed *CDR1* and *CDR2* (p-value > 0.0001).

### *MDR1* efflux pump overexpression

*MDR1* expression were not significantly different among S (MYA2876), SDD (ΔMYA4440) and R (ATCC96901) strains (p-value > 0.0001) (Fig. 1c). Without the addition of FLC, some SDD, R and some of S strains including 3 S reference strain and some S clinical strains expressed *MDR1*. After incubation with 8 μg/ml of FLC for 24 h, almost strains were not significantly changed in *MDR1* expression. Some strains (Y59, S13, G189, H282, G627, G1002, R237, S261 and G582) were observed MDR1 overexpression. Nonetheless, except 3 strains (Y4, Y5 and Y63) showed significant result as *MDR1* repression (p-value < 0.0001).

### *ERG11* target enzyme overexpression

Figure 1d explained *ERG11* overexpression. No statistically significant differences of *ERG11* expression (p-value > 0.0001) were seen among all strains. After incubation with 8 μg/ml of FLC for 24 h, all strains showed increased *ERG11* expression. The 15 strains of clinical susceptible strains (55.56%) were significantly increased *ERG11* expression (p-value < 0.0001), while 12 strains (44.44%) and all control strains were not significantly different (p-value > 0.0001).

### The protein spectrum related to the fluconazole resistance of *C. albicans* in control standard strains

This experiment was to observe FLC mass spectral change among S, SDD and R control strains after incubation with 4 and 8 μg/ml for 6 h. Before initiating the experiment, the quality control of reagent, microorganisms, drug, and incubation time period was investigated as shown in Fig. 2 and Fig. 3. Figure 2 showed the mass spectral range 0-600 m/z of 0.1%HCCA, FLC S *C. albicans* strain, FLC SDD *C. albicans* strain, FLC R *C. albicans* strain and FLC. The mass spectral intensity of FLC was appeared the spectrum position at 307 m/z, while those of 0.1%HCCA and FLC S, SDD and R strains was not seen any peak like FLC mass spectrum. After testing FLC incubation for 2, 4 and 6 h, the mass spectral intensity of FLC was

present all the time of study period (Fig. 3). This advocated that the incubation of studied time period does not affect the FLC mass spectrum. After testing with S and R *C. albicans* strains by incubation with 4 and 8 μg/ml for 6 h, FLC mass spectral intensity of 8 μg/ml FLC addition was higher than that of 4 μg/ml FLC addition. No FLC hydrolysis was detected in this experiment (Fig. 4). Spectrum position at 307 m/z of fluconazole can found in every fluconazole addition condition which is at 8 μg/ml of fluconazole concentration, the intensity will higher than at 4 μg/ml of fluconazole concentration (Fig. 4). This suggested that FLC hydrolysis is not the mechanism of action of FLC in *C. albicans*. Therefore, we cannot use FLC mass spectrum position to determine among FLC S, SDD and R strains.

Then, the spectrum pattern of *C. albicans* at mass spectral range 2,000-20,000 m/z was investigated after incubation with 0 (control), 4 and 8 μg/ml for 6 h (Fig. 5). Interestingly, the change of mass spectral intensity at range 3376-3382 m/z (major peak) was significantly related to the FLC susceptibility to S, SDD and R reference strains. Compared to 0 μg/ml of FLC, 3 types of major peak were: 1) increased 4 and 8 μg/ml (S strain), 2) decreased at 4 μg/ml and increase at 8 μg/ml (SDD strain), and 3) slightly decreased or no change at 4 and 8 μg/ml (R strain). After observing the major peak of all 27 clinical strains, their results showed S pattern like S reference strain (data not shown). This is in line with FLC MIC recognized as susceptible (MIC range 0.25-2 μg/ml) and no change in *CDR1* and *CDR2* overexpression.

# Discussion

Here, all *C. albicans* strains compared to MIC level in this study, *CDR1* and *CDR2* genes expression are only related to FLC SDD (ΔMYA4440) and R strains (ATCC96901) without FLC addition. These results disclosure the resistance mechanism via increased efflux of the drug from cells by ABC transporters. The R strain has a higher expression of *CDR1* and *CDR2* than SDD strain. Although only 2 samples including 1 spontaneous mutant SDD strain and 1 R reference strain are available in this study, the results are in line with previous reported that high expression levels of *CDR* efflux genes is a major mechanism for fluconazole resistance in *C. albicans* [6]. *MDR1* expression in our study was not significantly different among S, SDD and R strains (p-value > 0.0001). This may support the previous study revealed that in planktonic cell *CDR* displays higher expression level after 24 h incubation, while *MDR1* expression was even higher after 48 h [10]. Contradictory to our study revealed that *CDR1* overexpressed in SDD and R strains after 24 h incubation with FLC, previous study observed that FLC could induce the expression of *CDR1, CDR2* and *MDR1* [10]. For *ERG11* expression, all strains showed increased after incubation with 8 μg/ml FLC for 24 h. This supported the previous report that the expression of *ERG11* were increased after antifungal treatment in susceptible and non-susceptible to azole isolates [11]. Considering to determine FLC susceptibility to *C. albicans* with MALDI-TOF MS, we found only one spectrum at position 3376–3382 m/z that was linked with the FLC resistance of *C. albicans* after incubation with 4 and 8 μg/ml FLC for 6 h. Indeed, by allowing a peak position's tolerance of ± 3 m/z were overcome the small spectral variations and able to assess as *C. albicans* housekeeping peaks [12]. The drug concentration and incubation time period seem be a major factor for each *Candida* species to observe a major peak for differentiating S strain from R strain. In view of the study of Paul, both the resistant and susceptible *C. tropicalis* isolates showed spectral changes after 4 h when challenged with 128 μg/ml and 1 μg/ml FLC,

respectively [13]. In our study, the major mass spectral intensity change was clearly seen after 6 h incubation with FLC. As reported by Vatanshenassan using MBT ASTRA prototype software, the discrimination between resistant and susceptible strains was accurately detected in 6 h [14]. Furthermore, the previous report explained that prolonged exposures antifungal drug in some isolates were allowed to increasing correctly identifying between S and R strains [8]. Together with the condition of drug concentration and incubation time period, the method processed in each study could support for determining FLC resistant *C. albicans* strains and also finding different major mass spectrum. For example, the study of Marinach [15], MPCC determination was used to decide FLC *C. albicans* resistance strains with MALDI-TOF MS. Their study was concluded that the most suitable of FLC concentrations were 2 and 4 μg/ml and significant spectral intensity range 5,800–7,600 m/z were used to detect FLC resistance. Six spectrums in this range could detect the different of FLC resistance. Many studies used the difference of the operating procedure including MPCC method [13, 15], composite correlation index (CCI) values approach [8, 16, 17], matrix-assisted laser desorption ionization Biotyper antibiotic susceptibility test rapid assay (MBT-ASTRA) [1, 9] and machine-learning algorithms with the most robust pipeline of analysis [12]. Now, we disclosure a specific mass spectrum for analyzing FLC S, SDD and R strain for *C. albicans* with their resistance related to *CDR* expression efflux pump. For approving our results followed by the same protocol, more 27 *C. albicans* strains from clinical samples at various sites of 27 patients were investigated the major peak type by MALDI-TOF MS and FLC MIC by BMD. All strains presented a novel mass spectral intensity at range 3376–3382 m/z (major peak) and the major peak type was observed as S type and interpreted MIC level as susceptible (data not shown). Unfortunately, we could not find FLC SDD or R *C. albicans* strain from blood cultures of candidemic patients in the University Hospitals during our study. Moreover, we followed the protocol for determining in 48 *C. tropicalis* strains including FLC S, SDD and R strains from blood cultures of 48 patients. Unsuccessfully, the major peak type could not use for differentiating *C. tropicalis* (data not shown). With this reason, this major peak type is specific for determining FLC susceptibility testing to *C. albicans*.

# Conclusions

Overall, we discover a novel mass spectral intensity at range 3376–3382 m/z (major peak) related to the FLC susceptibility to S, SDD and R strains specific for *C. albicans*. The FLC resistance mechanisms of *C. albicans* associated with *CDR1* and *CDR2* expression (via increased efflux of the drug from cells by ABC transporters) may possibly effect the change of mass spectral intensity at range 3376–3382 m/z. The results demonstrate that the MALDI-TOF MS may be used to simultaneously classify *Candida* species and predict FLC resistant *C. albicans* strains associated with *CDR1* and *CDR2* overexpression. However, the limitations of our study including the small number of clinical *C. albicans* strains, the shortage of SDD and R clinical *C. albicans* strains, and unproven the protein spectrum at position 3376–3382 m/z. Therefore, further studies are essential to clarify the methodology and improve the reliability of this assay for routine diagnosis.

# Abbreviations

*C. albicans*: *Candida albicans*; MALDI-TOF MS: Matrix-assisted laser desorption ionization-time of flight mass spectrometry; Real-time PCR: Real-time polymerase chain reaction; *CDR*: *Candida* drug resistance; *MDR*: multidrug resistance; *ERG*: ergosterol biosynthesis; FLC: Fluconazole; S: Susceptible; R: Resistant; SDD: Susceptible-dose dependent; mRNA: messenger ribonucleic acid; MIC: minimal inhibitory concentration; ABC transporters: ATP-binding cassette transporters; MFS: Major facilitator superfamily; BMD: broth microdilution; AFST: antifungal susceptibility test; CLSI: Clinical and Laboratory Standards Institute; EUCAST: European Committee on Antibiotic Susceptibility Testing; SYO: Sensititre YeastOne; CBPs: clinical breakpoints; ECVs: epidemiological cutoff values; MPCC: minimal profile changing concentration; CCI: Composite correlation index; MBT-ASTRA: matrix-assisted laser desorption ionization Biotyper antibiotic susceptibility test rapid assay; SDA: Sabouraud Dextrose Agar; RPMI 1640 medium: Roswell Park Memorial Institute 1640 culture medium; cDNA: complementary deoxyribonucleic acid; qRT-PCR: quantitative real-time polymerase chain reaction; YNB: yeast nitrogen base; OD: optical density; RT-qPCR: quantitative reverse transcription polymerase chain reaction; RT inactivation: reverse transcriptase inactivation; *PMA1*: plasma membrane ATPase pump; DEPC: Diethyl pyrocarbonate; HPLC: high performance liquid chromatography; RT: room temperature; TFA: Trifluoroacetic acid; HCCA: α-Cyano-4-hydroxycinnamic acid; BTS: bacterial test standard

# Declarations

### Ethics approval and consent to participate

The research was approved by Human Ethical Committee of Thammasat University (054/2561) and Navamindradhiraj University (04/2018) and also accredited by Biosafety Committee of Thammasat University (038/2561).

### Consent for publication

Not applicable.

### Availability of data and material

All the data required is included in the manuscript.

### Competing interests

The authors declare that they have no competing interests.


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### Authors' contributions

Chanika Maenchantrarath carried out the study design, sample preparation, experiments, and data analysis and wrote the initial draft of the manuscript. Pradchama Khumdee helps some parts of MALDI-TOF MS experiment. Seksun Samosornsuk gave guidance and support. Narissara Mungkornkaew identified and collected some yeast samples. Worada Samosornsuk gave advices, contributed to the analysis of the evaluation of the study, and was a major contributor in writing the manuscript. All authors read and approved the final manuscript.

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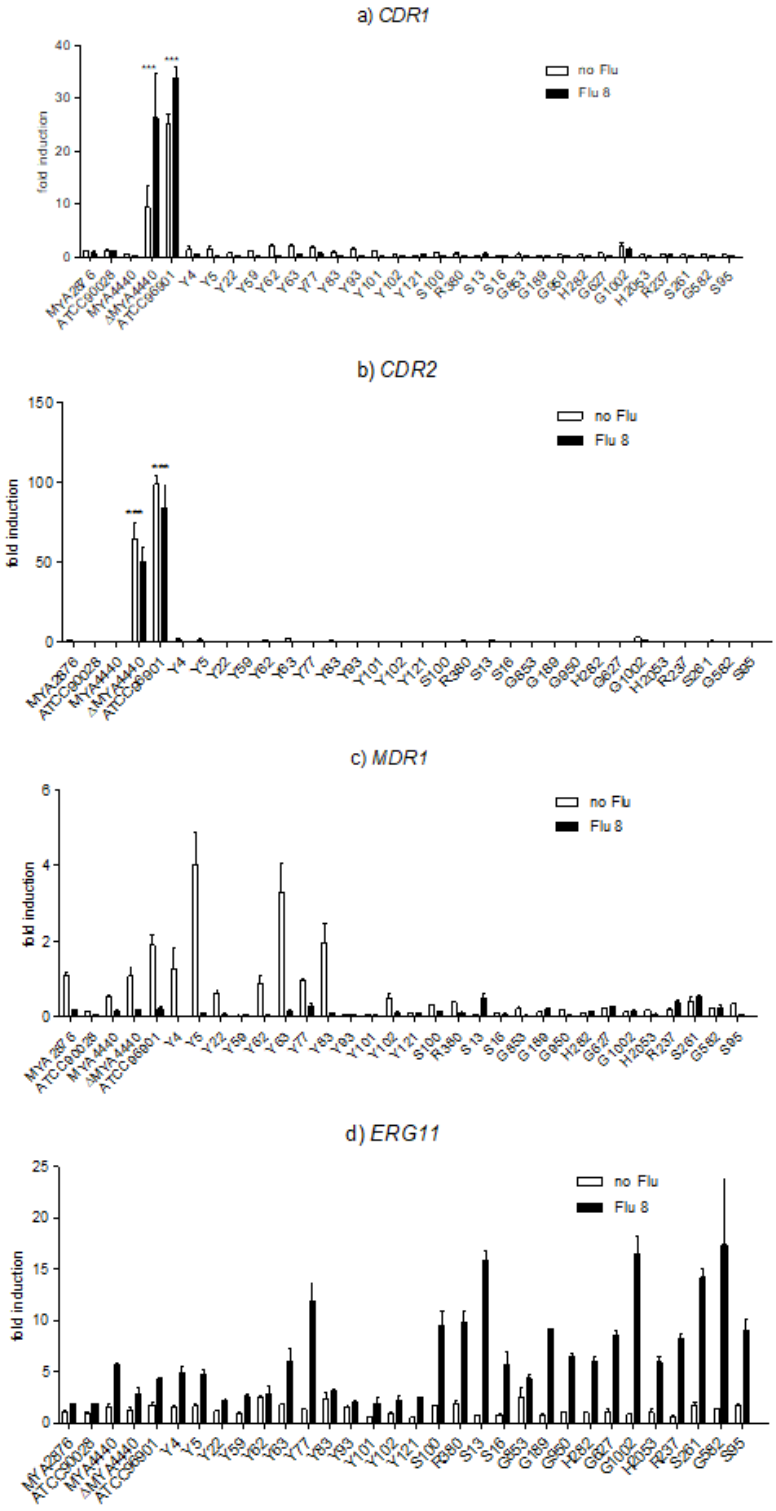
# Tables

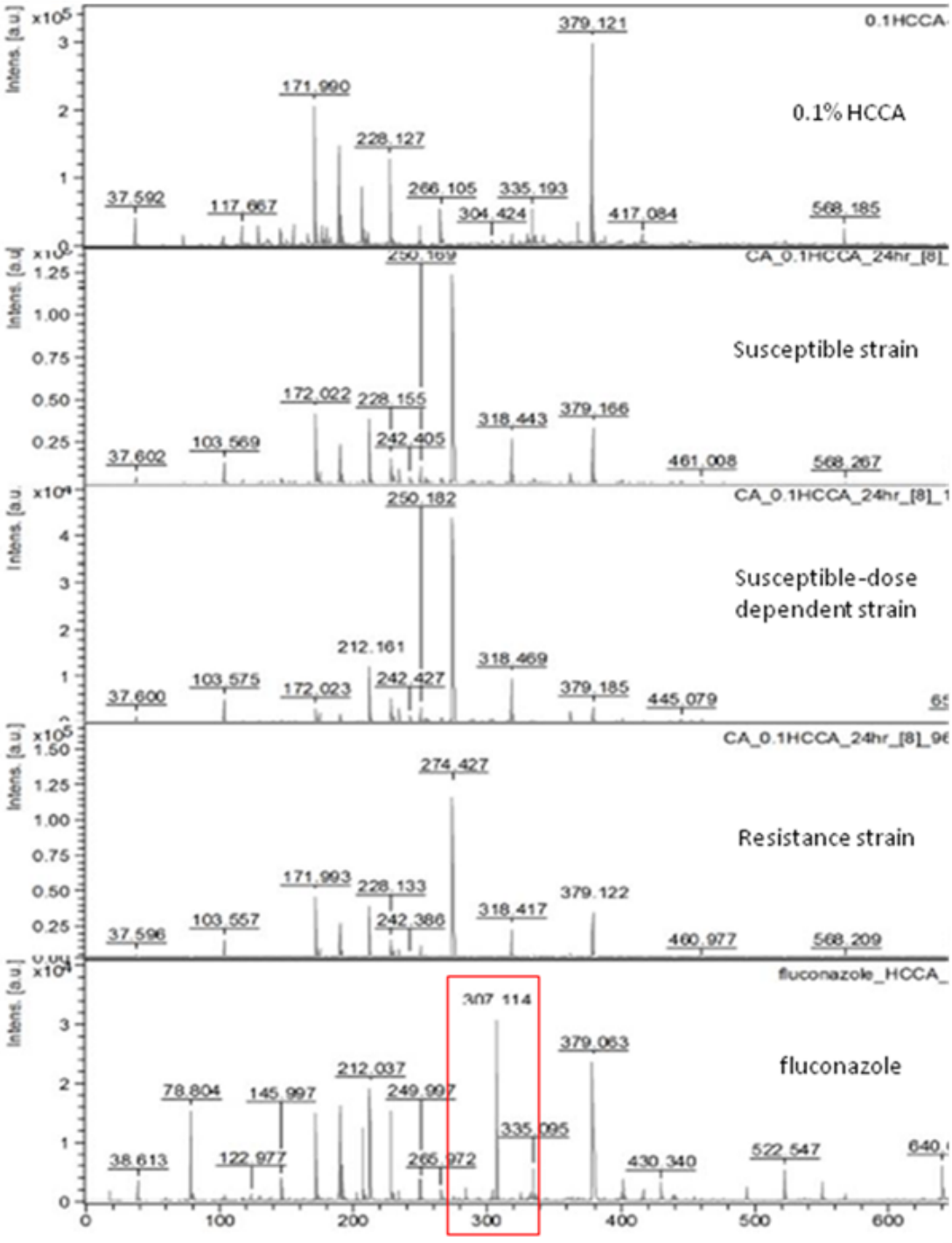
Due to technical limitations, table 1-3 is only available as a download in the Supplemental Files section.

# Figures



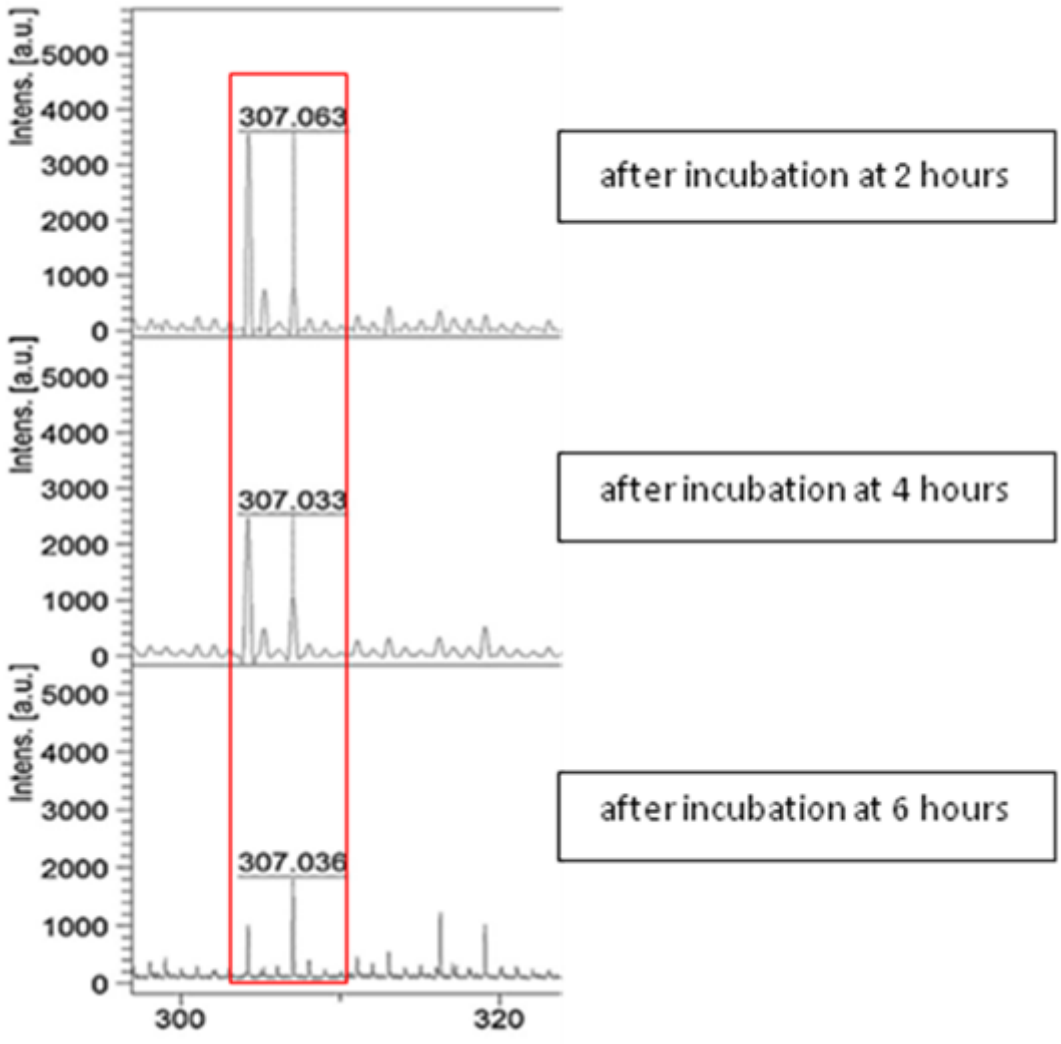

## Figure 1

The mRNA expression of target genes a) CDR1 b) CDR2 c) MDR1 and d) ERG11 in C. albicans susceptible strain (n = 30; MYA2876, ATCC90028, MYA4440, and 27 clinical strains), fluconazole susceptible-dose dependent strain (n = 1; ΔMYA4440) and resistant strain (n = 1; ATCC96901), comparison between without fluconazole condition and after fluconazole 8 μg/ml addition for 24 hours




## Figure 2

The spectrum of control 0.1% HCCA, fluconazole susceptible, susceptible-dose dependent strain, resistant C. albicans strains, and fluconazole position at 307 m/z (red box) (from top to bottom) using the reflector mode




## Figure 3

The spectrum of fluconazole position at 307 m/z (red box) after incubation at 2, 4 and 6 hours (from top to bottom) using the reflector mode

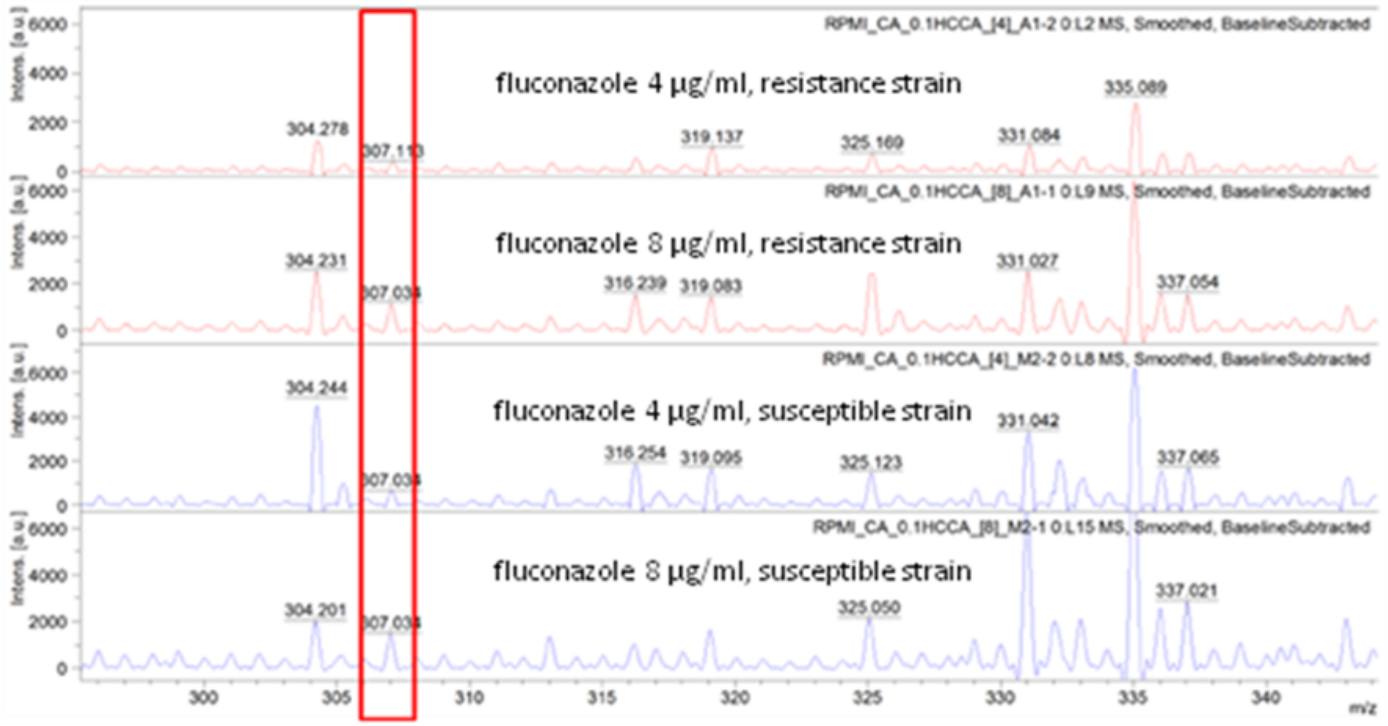



**Figure 4**

The comparison of fluconazole spectrum, position at 307 m/z (red box), after incubation at fluconazole concentration 4 and 8 µg/ml of resistance strain and susceptible strain, respectively (from top to bottom)

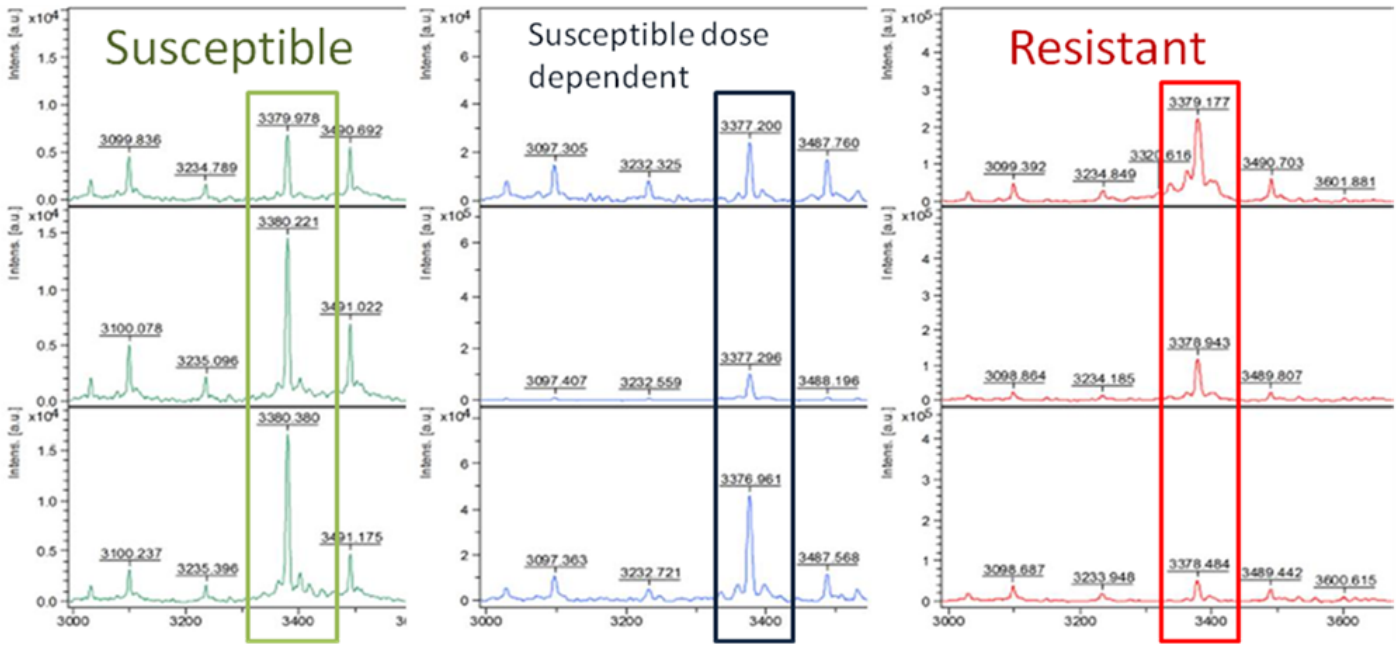



**Figure 5**

The spectrum intensity change at position 3376-3382 m/z of fluconazole-susceptible (green box) fluconazole-susceptible-dose dependent (blue box) and fluconazole-resistant (red box) C. albicans strains.

## Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- MaenchantrarathTable1.pdf
- MaenchantrarathTable2.pdf
- MaenchantrarathTable3.pdf