In Vitro Interaction between Fluconazole and Triclosan against Clinical Isolates of Fluconazole-Resistant Candida albicans Determined by Different Methods

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Running title: Interaction between TCL and FLC against C. albicans

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The in vitro interaction between triclosan and fluconazole against 24 azole-resistant clinical isolates of *Candida albicans* was evaluated by the microdilution checkerboard technique. The synergisms were verified by time-killing curves and agar diffusion tests in selected strains. Antagonistic activity was not detected.

**Keywords:** Resistant *Candida albicans*; Antifungal susceptibility; Antifungal interactions

*Candida albicans* is the primary cause of opportunistic fungal disease in humans. It is predominantly found at low levels among the normal oral flora but can thrive in immunocompromised individuals (16, 25). Fluconazole has been used successfully as a prophylactic and a first-line therapeutic antifungal agent (5, 6, 19). However, the increase in azole use has precipitated a rise in drug resistance in clinical isolates. Triclosan, a chlorinated aromatic compound, has antimicrobial (4, 8, 20), antiparasitic (26) and anti-inflammatory activities (1, 24). It has been used in personal care products (2). Combination therapy can improve the efficacy of antimicrobial therapy for infections recalcitrant to most treatments. Therefore, we aimed to assess the presence of combination effects with triclosan and fluconazole in *C. albicans*.

Twenty-four clinical isolates of fluconazole-resistant *C. albicans* were used in this study, and *C. albicans* ATCC 10231, *C. parapsilosis* ATCC 90018, and *C. krusei* ATCC 6258 were used as quality controls. The drugs were purchased from Sigma (Sigma–Aldrich, USA).

The drug MICs were determined by broth microdilution according to the CLSI
method M27-A (3) with an inoculum of $2.5 \times 10^3$ CFU/ml. The plates were incubated at 35°C, and the optical density (OD) value was determined at 492 nm after 48 h, a modification to the CLSI reference method. All experiments were conducted in triplicate, and the median MIC-1 endpoint, which represents an 80% reduction in turbidity, and MIC-2 endpoint, which represents a 50% reduction in turbidity, values were calculated (3). The drug interactions were analyzed using the FICI and $\Delta E$ models based on the Loewe additivity and Bliss independence theories, respectively (21, 14). The FICI were defined as the sum of the MIC of each drug used in the combination divided by the MIC of the drug used alone. Synergy and antagonism were defined by FICIs of $\leq 0.5$ and $>4$, respectively (15). The $\Delta E$ model was calculated as the sums of the percentages of all SS (statistically significant) synergistic ($\sum \text{SYN}$) and antagonistic ($\sum \text{ANT}$) interactions. Interactions that were $<100\%$ or $>200\%$ SS interactions were considered weak and strong, respectively. Interactions that were 100-200% SS interactions were considered moderate (14). The number of SS synergistic and antagonistic combinations was calculated for each strain.

One hundred microliters of $10^6$ CFU/ml C. albicans YL345, which exhibited the best synergistic effect, was spread onto a yeast extract-peptone-dextrose agar surface. Subsequently, 6-mm paper disks, impregnated with drugs or DMSO alone, were placed onto the surface. The inhibition zones were measured using a dial caliper after a 48-h incubation at 35°C. The tests were performed in duplicate (9, 18).

The time-kill curves were conducted in an RPMI 1640 medium with $10^5$ CFU/ml C.
*C. albicans* YL345. At different time points after the drug incubation, 100 µl of the tube contents was subcultured in serial dilutions (10⁻¹, 10⁻², 10⁻³, and 10⁻⁴) on Sabouraud dextrose agar plates. Colony counts were determined after a 48-h incubation at 35°C. The results were reported as the mean ± standard deviation of all three replicates conducted for each compound, alone and in combination. The synergism and antagonism were defined as respective increases or decreases of ≥ 2 log₁₀ CFU/ml in antifungal activity produced by the drug combination compared with the more active agent alone after 24 h (10, 12).

The checkerboard results are summarized in Table 1. The MIC-2 endpoint values for fluconazole and triclosan in *C. albicans* ranged from 4 to 32 µg/ml and from 32 to 64 µg/ml, respectively. The drug combination markedly reduced the MIC-2 endpoints of fluconazole and triclosan to 1 to 2 µg/ml and 4 to 8 µg/ml, respectively. Previous report has stated that the in vivo triclosan concentration in saliva was about 13 µg/ml at 10 min after brushing with toothpaste, and the duration of activity of triclosan at concentration of 10 µg/ml in saliva was about 0.7 hour (13). From our data, the value of MIC-2 of triclosan against azole-resistant *C. albicans* strains were 4 to 8 µg/ml when it combined with fluconazole. The concentration of triclosan ranged from 10 to 13 µg/ml in saliva was adequate to inhibit azole resistant strain when the two drugs were combined.

The corresponding median FICI and ∆E values are shown in Table 2. The FICIs ranged from 0.125 to 0.375 and from 0.125 to 0.25 when analyzed using the MIC-2 and the MIC-1 endpoints, respectively. The ∆E values ranged from 116.8% to 589.2%
when calculated using the MIC-2 endpoint. Antagonisms were not observed.

The synergism between fluconazole and triclosan was confirmed by agar diffusion tests (Fig. 1). The halo diameters produced by the combination were predominantly larger than ones produced by single drug treatments. The sizes of the inhibition zones increased to 19.2, 18, 15.6, and 10.8 mm when 16 µg/ml fluconazole was combined with 16, 8, 4, and 2 µg/ml of triclosan, respectively.

The time-kill curves verified the synergic combinations (Fig. 2). Triclosan and fluconazole did not significantly affect the isolate growth when the drugs were used alone at 16 µg/ml and 4 µg/ml, respectively. The combination therapy yielded a 3.0-log_{10}-CFU/ml decrease compared with triclosan alone after 24 h, wherein there was a significant difference (P < 0.01).

Taken together, our findings indicate that triclosan exhibits an antifungal effect in vitro against azole-resistant *C. albicans* when combined with fluconazole. In the checkerboard assay, the FICI model has been frequently used to determine the interaction between antifungal drugs (7, 9, 12, 17, 21, 23). The ∆E model is a useful method for characterizing drug interactions. We verified the positive interactions using the agar diffusion test and time-kill curves, which were able to detect differences in the rate and degree of antifungal activity over time (11). An agar diffusion test can provide more visually convincing results. A combination treatment with triclosan has been previously demonstrated to significantly enhance the efficacy of triclosan against microbes (20, 22). In contrast to various previous reports (20, 22), triclosan is a better synergist to fluconazole against *C. albicans*. 
In conclusion, the combination treatment of fluconazole and triclosan effectively synergizes against *C. albicans*. Our findings may provide an alternative approach to overcoming antifungal drug resistance. However, the mechanisms underlying the synergy must be further elucidated.

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**REFERENCE**


## Figure legends

### Table 1
The in vitro interaction between TCL and FLC against 24 clinical isolates of *C. albicans*

<table>
<thead>
<tr>
<th>Drug</th>
<th>Median MIC-2 endpoint (range) of drug (µg/ml)</th>
<th>Median MIC-1 endpoint (range) of drug (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Alone</td>
<td>In combination</td>
</tr>
<tr>
<td>FLC</td>
<td>16 (4-32)</td>
<td>1 (1-2)</td>
</tr>
<tr>
<td>TCL</td>
<td>32 (32-64)</td>
<td>8 (4-8)</td>
</tr>
</tbody>
</table>

TCL, triclosan; FLC, fluconazole.

### Table 2
The in vitro interaction between TCL and FLC against clinical isolates of *C. albicans*

<table>
<thead>
<tr>
<th>Strain</th>
<th>FICI model Median (range)</th>
<th>∆E model Median (range)</th>
<th>∑SYN % (±)</th>
<th>∑ANT % (±)</th>
<th>NT</th>
<th>∑SYN % (±)</th>
<th>∑ANT % (±)</th>
<th>INT</th>
</tr>
</thead>
<tbody>
<tr>
<td>24</td>
<td>0.31 (0.125)</td>
<td>0.25 (0.125)</td>
<td>SYN</td>
<td>-28.3% (5)</td>
<td>SYN (all isolates)</td>
<td>90.1</td>
<td>90% to</td>
<td>-8.3% (2)</td>
</tr>
<tr>
<td>isolates</td>
<td>to 0.377</td>
<td>to 0.25</td>
<td>(all isolates)</td>
<td>to 0.25</td>
<td>(all isolates)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

TCL, triclosan; FLC, fluconazole. INT, Interpretation; SYN, synergism; ANT, antagonism; IND, indifference; n, number of interactions. *The FICI and ∆E models determined based on MIC-2 endpoint; †, The FICI and ∆E model determined based on MIC-1 endpoint.*
Figure 1. The agar disk diffusion assay for FLC combined with TCL in *C. albicans* YL345. Panel B describes the image for panel A, and panel D describes the image for panel C.

Figure 2. The time-kill curves for FLC and TCL alone and in combination in clinical azole-resistant *C. albicans* YL345. ●, growth control; ■, FLC; ▲, TCL; *, FLC plus TCL. TCL, triclosan; FLC, fluconazole.