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Multiple effects of green tea catechin on the antifungal activity of antimycotics against *Candida albicans*

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Objectives: The susceptibility of *Candida albicans* to catechin under varying pH conditions and the synergism of the combination of catechin and antimycotics were evaluated.

Method: Antifungal activity was determined by broth dilution and calculation of cfu.

Results: The antifungal activity of catechin was pH dependent. The concentration of epigallocatechin gallate (EGCg) causing 90% growth inhibition of tested strains of *C. albicans* was 2000 mg/L at pH 6.0, 500–1000 mg/L at pH 6.5 and 15.6–250 mg/L at pH 7.0. Among catechins, pyrogallol catechin showed stronger antifungal activity against *C. albicans* than catechol catechin. The addition of 6.25–25 or 3.12–12.5 mg/L EGCg to amphotericin B 0.125 or 0.25 mg/L (below MIC) at pH 7.0 resulted in enhancement, respectively, of the antifungal effect of amphotericin B against amphotericin B-susceptible or -resistant *C. albicans*. Combined treatment with 3.12–12.5 mg/L EGCg plus amphotericin B 0.5 mg/L (below MIC) markedly decreased the growth of amphotericin B-resistant *C. albicans*. When fluconazole-susceptible *C. albicans* was treated with 25–50 mg/L EGCg and fluconazole 0.125–0.25 mg/L (below MIC), its growth was inhibited by 93.0%–99.4% compared with its growth in the presence of fluconazole alone. The combined use of 12.5 mg/L EGCg and fluconazole 10–50 mg/L (below MIC) inhibited the growth of fluconazole-resistant *C. albicans* by 98.5%–99.7%.

Conclusions: These results indicate that EGCg enhances the antifungal effect of amphotericin B or fluconazole against antimycotic-susceptible and -resistant *C. albicans*. Combined treatment with catechin allows the use of lower doses of antimycotics and induces multiple antifungal effects. It is hoped that this may help to avoid the side effects of antimycotics.

Keywords: Japanese green tea, polyphenols, antifungal effects, yeast

Introduction

Candida albicans is part of the indigenous microbial flora in humans and can be found in the oral cavity and the digestive and vaginal tracts, and is unique among opportunistic pathogens because it is part of the normal microbial flora of the host.[1] However, an increased prevalence of candidosis is well documented and has been attributed to the widespread use of antibiotics and immunosuppressive agents.[2] *C. albicans* has been shown to play an important role in oral candidosis, denture stomatitis and severe periodontitis.[3–6]

Amphotericin B is one of the polyene antibiotics, and fluconazole is an azole antifungal agent. They have strong antifungal activity, especially against *C. albicans*. However, they also have side effects,[7] and antimycotic-resistant clinical isolates of *C. albicans* have appeared.[8,9] Therefore, a non-antibiotic agent that is both highly effective and safe might be important for the eradication of both antibiotic-susceptible and -resistant strains of *C. albicans*. There are several reports that show antifungal activity by natural products.[10–14] Green tea is a natural substance that is commonly drunk worldwide, especially in Asia. Catechin from tea has been reported to have an antimicrobial effect against oral,[15,16] intestinal[17] and food-borne[18] bacteria, antitoxicity against various bacterial haemolysins[19] and antiviral activity.[20]

In this study, we examined the antifungal effects on *C. albicans* of tea catechins on their own and combined with antimycotics.

Materials and methods

Microorganisms and culture conditions

Candida albicans ATCC 90028, ATCC 90029, ATCC 96901 and ATCC 200955, and 10 clinical isolates were used in this study. All strains were

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Table 1. MIC$_{90}$ and MFC of EGCg for *C. albicans* at various pHs

	MIC$_{90}$ and MFC (mg/L)							
	ATCC 90028		ATCC 90029		ATCC 96901		ATCC 200955	
pH	MIC$_{90}$	MFC	MIC$_{90}$	MFC	MIC$_{90}$	MFC	MIC$_{90}$	MFC
6.0	2000	8000	2000	8000	2000	4000	2000	8000
6.5	1000–500	2000	1000–500	2000	500	2000	500	2000
7.0	250–62.5	2000	125–31.2	1000	15.6	250–125	62.5–31.2	1000–500

maintained routinely and cfu were calculated on Sabouraud agar (Nissui Co., Tokyo, Japan). The plates were incubated aerobically at 37°C for 48 h.

Catechins and antimycotics

The catechins used in this study were (–)-epigallocatechin gallate (EGCg), (–)-epicatechin gallate (ECg), (–)-epigallocatechin (EGC), (–)-epicatechin (EC), (+)-catechin (C), catechin gallate (Cg), (+)-gallocatechin (GC) and gallocatechin gallate (GCg), and they were purchased from Funakoshi Co. (Tokyo, Japan). Amphotericin B and fluconazole were purchased from Sigma Chemical Co. (St Louis, MO, USA).

Measurement of MIC of EGCg at various pHs

Measurement of the MIC of EGCg for *C. albicans* was performed by broth dilution and calculation of cfu. In the experiments, RPMI medium 1640 (Gibco BRL) buffered with 0.15 M sodium phosphate buffer (NaPB-RPMI) at pH 6.0, 6.5 or 7.0 was used for the test medium. The experimental medium was prepared by twofold dilution of 8000 mg/L EGCg with NaPB-RPMI at pH 6.0, 6.5 or 7.0. *C. albicans* was pre-incubated in the NaPB-RPMI and buffered at each pH at 37°C for 24 h with shaking (100 strokes/min). The pre-cultured *C. albicans* (final fungal count of ~1 × 10^3 cfu/mL) was inoculated into 1 mL of the experimental media at various pHs. After cultures were shaken at 37°C for 48 h, they were spread on Sabouraud agar plates at 10-fold dilutions in triplicate. The plates were incubated at 37°C for 48 h under aerobic conditions. Antifungal activity was determined by calculation of cfu. The minimum concentration that inhibited the growth of *C. albicans* on the Sabouraud agar plates by 90%, compared with the growth in EGCg-free medium, was defined as the MIC$_{90}$.[10] The minimum fungicidal concentration (MFC) was determined as the lowest concentration resulting in the death of 99.9% or more of the initial inoculum. To determine MFCs, 0.1 mL of the test sample was inoculated on Sabouraud agar plates in triplicate and incubated at 37°C for 48 h. The cfu were counted to assess viability.[10]

Assay of antifungal activity of catechins

To investigate the effect of catechins on non-multiplying fungal cells, resting fungal cells were prepared. *C. albicans* was cultured in brain heart infusion (BHI, Difco Laboratories, Detroit, MI, USA) broth at 37°C for 24 h aerobically with shaking. The growing cells were harvested, washed three times with 0.15 M NaPB (pH 7.0), suspended in the same buffer to a final concentration of ~1 × 10^6 cfu/mL and used for the assay. One milligram per mL of each catechin was added to the resting cells (~1 × 10^6 cfu/mL) in NaPB, pH 7.0, and the mixture was incubated at 37°C with shaking. Aliquots (0.1 mL) of the cell suspensions were collected over an extended period. Ten-fold dilutions of the samples were made in 0.9 mL of Tris–HCl buffer (0.05 M, pH 7.0) and inoculated onto

Sabouraud agar plates. The plates were cultured aerobically at 37°C for 48 h and the cfu calculated.

Measurement of multiple effects of EGCg and antimycotics

Assays of antifungal activity against *C. albicans* were performed in a similar manner to that described above for the measurement of MIC. Catechins, amphotericin B and fluconazole were prepared at 50–3.12, 0.5–0.125 and 50–0.125 mg/L in NaPB-RPMI at pH 7.0. At these concentrations, none of the agents alone affects the growth of *C. albicans*. The pre-cultured *C. albicans* (~2 × 10^7 cfu/mL) was adjusted to ~2 × 10^3 cfu/mL with NaPB-RPMI at pH 7.0 using the 10-fold dilution method for inoculation. One millilitre of these diluted solutions of *C. albicans* was added to 1 mL of the mixtures of various concentrations of EGCg and antimycotic solutions in NaPB-RPMI at pH 7.0. After shaking incubation at 37°C for 48 h, the cultures were spread on plates at 10-fold dilutions in triplicate, and the cfu calculated. The percentage of growth inhibition was calculated from the cfu compared with that of drug-free control cultures.

Statistical analysis

Data shown are from three separate experiments and were analysed statistically by calculating means and S.D. of the means. The differences were evaluated by Student's *t*-test.

Results

Measurement of MIC

The MIC$_{90}$ and MFC of EGCg for *C. albicans* ATCC 90028, ATCC 90029, ATCC 96901 and ATCC 200955 are shown in Table 1. The antifungal effect of EGCg was dependent on pH. The MIC$_{90}$ of EGCg at pH 6.0 against ATCC 90028, ATCC 90029, ATCC 96901 and ATCC 200955 strains was 2000 mg/L. However, at pH 7.0 it was 15.6–250 mg/L. The MFC of EGCg against the test strains was 4000–8000 mg/L at pH 6.0 and 125–2000 mg/L at pH 7.0. Strain ATCC 96901, which is fluconazole resistant, was slightly more susceptible to EGCg than the other strains. Table 2 shows the MIC$_{90}$ of EGCg for clinical isolates of *C. albicans* at pH 7.0. The range of MIC$_{90}$ values was 31.2–250 mg/L. The MIC$_{90}$ of fluconazole and amphotericin B for strain ATCC 90029 at pH 7.0 was 0.5–1 mg/L and 0.25–0.5 mg/L, respectively (data not shown).

Antifungal activity of various catechins

Figure 1 shows the antifungal effects of the various catechins on resting fungal cells of *C. albicans* ATCC 90029. The survival of resting cells decreased immediately and rapidly with EGC, GC, EGCg and

Table 2. MIC_{90} of EGCg against clinical isolates of *C. albicans* at pH 7.0

Strain	MIC_{90} (mg/L)
NUM-CA11	125–62.5
NUM-CA12	125–31.2
NUM-CA18	125–31.2
NUM-CA22	250–62.5
NUM-CA26	62.5–31.2
NUM-CA27	125–31.2
NUM-CA35	125–31.2
NUM-CA39	62.5–31.2
NUM-CA43	125–62.5
NUM-CA48	62.5–31.2

Figure 2. The effect of the combination of amphotericin B (AMPH) with EGCg on the growth of *C. albicans* ATCC 90029. The culture was incubated in NaPB-RPMI, pH 7.0, for 48 h. Error bars indicate s.d. *Values differ significantly ($P < 0.01$) from values without catechin.

Figure 3. The effect of the combination of amphotericin B (AMPH) with EGCg on the growth of *C. albicans* ATCC 200955. *Values differ significantly ($P < 0.01$) from values without catechin.

Figure 1. Antifungal effect of various catechins against *C. albicans* ATCC 90029 using resting cells with NaPB, pH 7.0.

GCg, and the survival rate was <1% after 4 h. A few colonies still survived after 24 h of culturing. In contrast, the survival of resting cells treated with C, EC, Cg and ECg had hardly decreased after 4 h, but decreased gradually and slowly with increasing incubation time. The survival rate was 1%–10% after 24 h of culturing.

The antifungal effects of EC, EGC and ECg on resting cells of *C. albicans* ATCC 90028 and clinical isolates NUM-CA11, NUM-CA18, NUM-CA27, NUM-CA35 and NUM-CA43 were also examined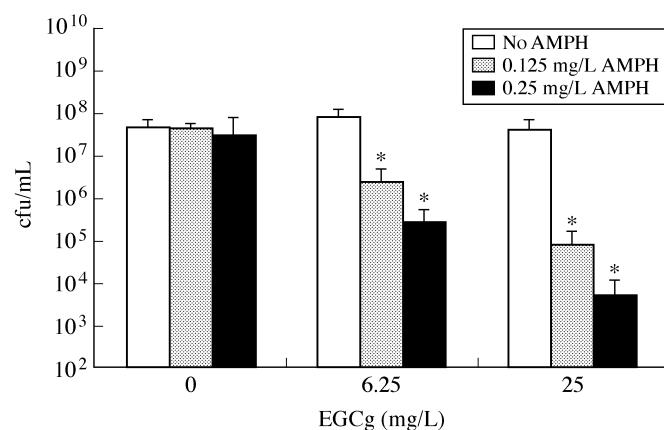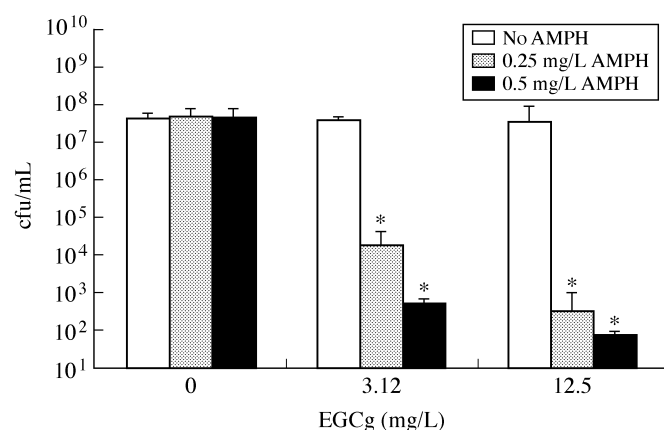. The effects of EC and ECg on ATCC 90028 and the five clinical isolates showed gradual antifungal activity similar to that on strain ATCC 90029. The effects of EGC on ATCC 90028 and the five clinical isolates were observed to be more marked than the effects of catechol catechins, similar to the effects on ATCC 90029 (data not shown).

Effect of EGCg on the antifungal activity of antimycotics

The addition of EGCg to amphotericin B resulted in enhancement of the antifungal activity of amphotericin B (Figures 2 and 3). Amphotericin B at 0.125 and 0.25 mg/L in the presence of 6.25 mg/L EGCg caused, respectively, 94.2% and 99.5% inhibition of the growth of amphotericin B-susceptible ATCC 90029. Stronger growth inhibition was obtained with 25 mg/L EGCg. The ATCC 90029 strain grew to only 0.2% or 0.01% of the extent seen with amphotericin B alone. The addition of 3.12 mg/L EGCg to amphotericin B 0.25 or 0.5 mg/L inhibited the growth of amphotericin B-resistant *C. albicans* ATCC 200955, resulting in, respectively, 0.04% or 0.001% of the extent of growth seen with amphotericin B alone. A stronger synergic effect of EGCg was obtained using 12.5 mg/L with ampho-
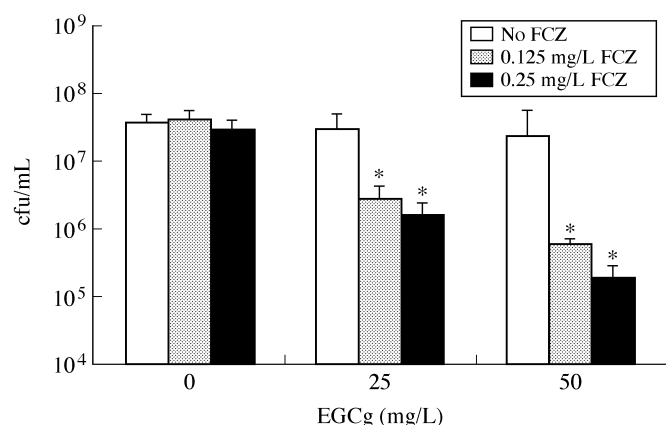
Figure 4. The effect of the combination of fluconazole (FCZ) with EGCg on the growth of *C. albicans* ATCC 90029. *Values differ significantly ($P < 0.01$) from values without catechin.
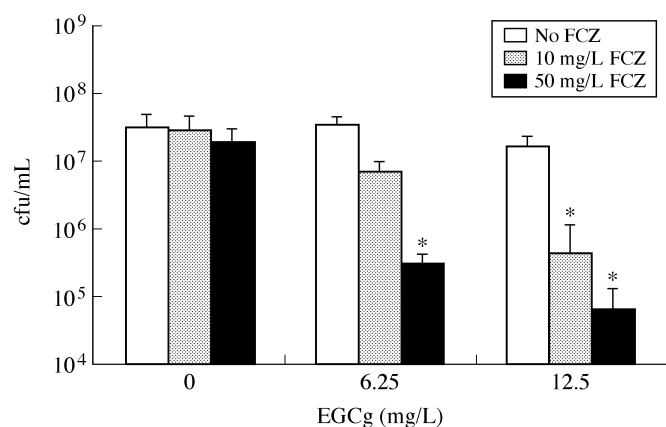

Figure 5. The effect of the combination of fluconazole (FCZ) with EGCg on the growth of *C. albicans* ATCC 96901. *Values differ significantly ($P < 0.01$) from values without catechin.

tericin B 0.25 and 0.5 mg/L. The growth level of the ATCC 200955 strain was 3.4×10^2 and 8.3×10 cfu at these concentrations, whereas that of the control was ~5×10^7 cfu.

The effect of the combined use of fluconazole and EGCg on antifungal activity against fluconazole-susceptible *C. albicans* is shown in Figure 4. The growth of ATCC 90029 was observed to be 93.0% and 95.1% inhibited using fluconazole 0.125 and 0.25 mg/L, respectively, together with 25 mg/L EGCg, compared with fluconazole alone. The strongest growth inhibition was obtained with the combined use of fluconazole 0.125–0.25 mg/L and 50 mg/L EGCg, with 98.5%–99.4% inhibition compared with controls.

Figure 5 shows the effect of the combined use of fluconazole and EGCg on fluconazole-resistant *C. albicans* ATCC 96901. The MIC$_{90}$ of fluconazole for the ATCC 96901 strain at pH 7.0 was 200 mg/L (data not shown). Strain ATCC 96901 grew well in the presence of less than fluconazole 50 mg/L. The combined use of fluconazole 10 mg/L and 6.25 or 12.5 mg/L of EGCg caused, respectively, 76.1% and 98.5% growth inhibition of the ATCC 96901 strain compared with fluconazole alone. Furthermore, the addition of 6.25 mg/L or 12.5 mg/L EGCg to fluconazole 50 mg/L caused, respectively,

98.4% and 99.7% growth inhibition compared with fluconazole alone.

To confirm these multiple effects further, similar examinations were performed using 25 mg/L EGCg and amphotericin B 0.125 mg/L or fluconazole 0.25 mg/L, concentrations at which neither agent alone affects the growth, and *C. albicans* ATCC 90028 and clinical isolates NUM-CA11, NUM-CA18, NUM-CA27, NUM-CA35 and NUM-CA43. The growth of ATCC 90028 and the clinical isolates was inhibited, respectively, 99.0%–99.9% and 95.1%–98.6% compared with antimycotic-free growth using the combination of EGCg and amphotericin B or EGCg and fluconazole (data not shown).

Discussion

It has been reported that tea catechins have antibacterial activity against various pathogenic bacteria.[15–18,21,22] Concerning fungi, Okubo *et al.*[23] reported that 2.5% of black tea extract completely inhibited the growth of *Trichophyton mentagrophytes* and *Trichophyton rubrum*; however, even at a 10% concentration, this extract did not inhibit the growth of *C. albicans* or *Cryptococcus (Filobasidiella) neoformans*. Recently, botanical,[10,13] marine[11,14] and bacterial[12] natural products were reported to have antifungal activity. In the present study, we showed that the antifungal activity of catechins against *C. albicans* was pH dependent (Table 1). These findings suggest that the antifungal action of EGCg was weakened by acidic conditions. The MIC$_{90}$ of EGCg increased by more than 10-fold as the pH was reduced from 7.0 to 6.5 and further increased several fold as the pH was reduced from 6.5 to 6.0.

For reference, a normal cup of tea has a concentration of ~1000 p.p.m. polyphenol. The catechins extracted from Japanese green tea consist of mainly EGCg, EGC and GC, and minor amounts of EC, ECg and C. Cg and GCg are not contained in extracts from green tea. Among these catechins, pyrogallol catechins (EGCg, EGC, GC and GCg) were more effective than catechol catechins (EC, ECg, C and Cg) against *C. albicans* (Figure 1). The actions of EGCg, EGC and GC were fungicidal. Studies of the antibacterial activity of catechins against phytopathogenic bacteria showed results similar to those against *C. albicans*.[22] Ikigai *et al.*[24] reported that the mechanism of the bactericidal effects of catechins primarily involved acting on and damaging bacterial membranes of *Staphylococcus aureus* and *Escherichia coli*. The antibacterial activities of catechins were predominantly related to the gallic acid moiety and the hydroxyl group member.[24] The mode of catechin action involves inducing rapid leakage of small molecules entrapped in the intraliposomal space and aggregation of the liposomes.[24] Toyoshima *et al.*[25] examined the mechanism of the effects of green tea catechin on *T. mentagrophytes* using electron microscopy and suggested that catechin attacked the cell membrane and caused lysis of the conidia and hyphae.

The present study also showed synergic antifungal activity of the combination of EGCg and antimycotics against *C. albicans*. Amphotericin B possesses antifungal activity against *C. albicans*. However, amphotericin B has strong side effects even at low doses. The combination of amphotericin B and 5-fluorocytosine, an antimycotic, was tried in an attempt to reduce the effective dose of amphotericin B.[26] In the present study, the combined use of EGCg and amphotericin B (below MIC) inhibited the growth of *C. albicans*, and the action was fungicidal. Amphotericin B binds to ergosterol, one of the cell membrane sterols, and damages the cell membrane directly, leading to fungicidal activity against the fungi. Amphotericin B below the MFC also stimulates fungal membrane permeability. The combined use of amphotericin B and catechin may stimulate catechin

uptake into the cell by the action of amphotericin B, and intracellular catechin may act as a fungicidal agent. A similar finding was reported previously. Catechin induces antibacterial activity of oxacillin below the MIC against methicillin-resistant *S. aureus*.[27] The bactericidal mechanism may be as follows: first, catechin acts on and damages bacterial membranes, and second, oxacillin binds to penicillin-binding protein and deactivates bacteria.[24] Since the arrival of azole antifungal agents as first-line drugs,[28] fluconazole-resistant *C. albicans* has begun to appear.[8] The combined use of EGCg and fluconazole was effective even against fluconazole-resistant *C. albicans*. The effective dose of fluconazole was decreased to one-fortieth using 6.25 mg/L EGCg compared with the growth in the presence of fluconazole alone (Figure 5). *C. albicans* expresses multidrug efflux transporter (MET), which mediates the efflux of a broad range of compounds, including fluconazole. MET inhibitor, cyclosporine, and fluconazole showed a potent synergic effect against *C. albicans*.[29] The mechanism of the synergic effect of the combination of fluconazole and EGCg is still unknown.

C. albicans superinfection results from taking antibiotics for a long period, and is predominantly detected in the oral cavity, intestine and vagina. Local administration of amphotericin B and fluconazole is very effective against *C. albicans*. The addition of catechin to amphotericin B or fluconazole induces antifungal activity via stimulation of multiple functions (Figures 2–5). Catechins are not destroyed and retain their effectiveness when exposed to artificial gastric juice for over 60 min (data not shown). Catechin combined with amphotericin B or fluconazole, and perhaps other antimycotics, may be beneficial and may contribute to the effective medical treatment of candidoses, such as thrush, denture stomatitis, and intestinal candidosis. However, *in vivo* experiments would be needed to test these possibilities in living animals or humans.

Acknowledgements

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