

# Antifungal Synergy of Theaflavin and Epicatechin Combinations Against *Candida albicans*

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New antifungal agents are required to compensate for the increase in resistance to standard antifungal agents of *Candida albicans*, which is an important opportunistic fungal pathogen that causes minor infections in many individuals but very serious infections in those who are immune-compromised. In this study, combinations of theaflavin and epicatechin are investigated as potential antifungal agents and also to establish whether antifungal synergy exists between these two readily accessible and cost-effective polyphenols isolated from black and green tea. The results of disc diffusion assays showed stronger antibacterial activity of theaflavin:epicatechin combinations against *C. albicans* NCTC 3255 and NCTC 3179, than that of theaflavin alone. Minimum inhibitory concentrations (MICs) of 1,024 µg/ml with theaflavin and 128–256 µg/ml with theaflavin:epicatechin combinations were found. The fractional inhibitory concentration indexes were calculated, and the synergy between theaflavin and epicatechin against both isolates of *C. albicans* was confirmed. Theaflavin:epicatechin combinations show real potential for future use as a treatment for infections caused by *C. albicans*.

**Keywords:** Antifungal, theaflavin, epicatechin, synergy, *Candida albicans*

## Introduction

*C. albicans* is a common opportunistic pathogen, often part of the human microflora, causing minor infections, such as mucosal candidiasis, in healthy individuals [17] but also life-threatening infections in the immunocompromised (HIV, cancer) and critically ill [16]. Although effective antifungal treatments are available (polyenes, azoles, echinocandins), there are increasing reports of resistance in *Candida* species [5, 19] and an on-going need to develop new antifungal agents. Plant polyphenolic compounds extracted from tea leaves (*Camellia sinensis*) have been shown to exhibit a variety of natural health benefits in humans [9] and also to have antimicrobial properties. Epicatechin (EP) and theaflavin (TF) derivatives have been shown to have potentially useful antibacterial activity against gram-positive and gram-negative bacteria [6, 20] as well as yeasts and molds [14, 18]. Tea polyphenols have also been shown to influence biofilm formation by *C.*

*albicans* [4]. Enhanced activity, when polyphenols are tested either in combination or in the presence of other antimicrobials, has also been observed, and they may even suppress the emergence of resistance to existing antifungal drugs [3, 13]. In earlier studies, we identified synergy when EC and EF were combined *in vitro* against multidrug-resistant gram-negative bacteria [1, 2].

In this study we assessed whether combinations of the polyphenols epicatechin and theaflavin might be useful as potential antifungal agents against isolates of the potential pathogen, *Candida albicans*.

## Materials and Methods

*C. albicans* NCTC 3255 and NCTC 3179 were purchased from Pro-Lab Diagnostics, UK. ISO-sensitest broth and agar were purchased from Oxoid, UK. Blank susceptibility discs were purchased from Mast Group Ltd, UK. Samples of theaflavin (>95% purity) were donated by Unilever, China, and epicatechin (>90%

purity) was purchased from SigmaAldrich, UK. All media, agar, and blank susceptibility discs were autoclaved prior to their use.

#### Broth Microtiter Dilution Assays

A 4.10 mg/ml TF stock solution was prepared by dissolving 81.92 mg of theaflavin in 0.50 ml of dimethylsulfoxide (DMSO) before the addition of 19.50 ml of sterile ISO-sensitest broth. From this stock solution, a double dilution was performed eight times into ISO-sensitest broth to give solutions of TF with nine different concentrations (4.1–0.016 mg/ml). A 2.05 mg/ml EC stock solution was prepared by dissolving 40.96 mg of EC in 0.5 ml of DMSO before the addition of 19.50 ml of sterile ISO-sensitive broth. From this stock solution, a double dilution was performed five times into ISO-sensitest broth to give solutions of EC with six different concentrations (2.05–0.064 mg/ml). A fixed volume (50 µl) of TF and EC was pipetted into each well of a microtiter plate, whereby each row and column contained a fixed amount of one antimicrobial agent and increasing concentrations of the other antimicrobial agent, providing final well concentrations (including inoculum) of 4–1,024 µg/ml of TF and 16–512 µg/ml of EC. Control wells contained either 2.5% DMSO alone, or TF alone (4–1,024 µg/ml). Epicatechin alone (16–512 µg/ml) was also tested. A 100 µl volume of a  $5 \times 10^3$  colony forming unit (CFU)/ml suspension in ISO-sensitest broth was pipetted in order to inoculate each well with *C. albicans* NCTC 3255. This procedure was replicated for *C. albicans* NCTC 3179. All microtiter plates were incubated at 37°C for 24 h. A 3-fold replication of all experimental plates was undertaken, allowing the results to be provided as mean values.

Each well was observed for turbidity at the end of the incubation period. The lowest concentration at which no turbidity could be observed was taken as the MIC. Fractional inhibitory concentrations, indices (FICIs) were determined based on the method previously described [7], whereby the  $FIC_a = MIC$  of compound a + compound b/ $MIC$  of compound a, the  $FIC_b = MIC$  of compound b + compound a/ $MIC$  of compound b, and the  $FICIs = FIC_a + FIC_b$ . Where a FICI value was calculated to be  $\leq 0.5$ , synergy was recorded. A value  $>0.5$ –4.0 was noted as an additive effect and a value  $>4$  was considered an antagonism effect between theaflavin and epicatechin.

The MIC of epicatechin alone was also determined by microtiter dilution using wells containing 512–2,048 µg/ml for each of the *C. albicans* isolates. All microtiter plates were performed in triplicate and incubated at 37°C for 24 h.

#### Disc Diffusion Assay

A TF stock solution was prepared by adding 0.50 g of theaflavin powder to 5 ml of ethanol and stirring the resultant suspension for 10 min to provide a 100 mg/ml solution. A 100 mg/ml stock solution of epicatechin was prepared by gradually adding 0.05 g of epicatechin powder to 5 ml of ethanol and stirring for 15–20 min until the polyphenolic compound had dissolved. A combined solution of theaflavin and epicatechin (2:1) was prepared by adding 0.50 g of theaflavin and 0.25 g of epicatechin to 5 ml of

ethanol and stirring until both compounds had dissolved (15–20 min).

Polyphenol-susceptibility discs were prepared by pipetting 10 ml volumes of the individual polyphenol solutions directly onto blank discs. These were then dried for 20 min prior to the addition of any further solutions, if required. Single and combined discs (TF:EC) containing 2, 4, 6, and 8 mg of polyphenol were made by pipetting the appropriate volume of stock solution directly onto blank discs. Ethanol control discs were prepared using identical volumes of ethanol. All discs were then allowed to dry in a sterile Petri dish at room temperature, before their use, in order to completely remove all traces of solvent and thus eliminate any ethanol-mediated antifungal activity.

ISO-sensitive agar plates were independently inoculated with  $5 \times 10^3$  CFU/ml solutions of *C. albicans* [15] using the standardization method [12]. Plate 1 comprised discs containing 2, 4, 6, or 8 mg of epicatechin and an ethanol control disc. Plate 2 comprised discs with 2, 4, 6, or 8 mg of theaflavin and an ethanol control disc and separate discs containing 2, 4, 6, or 8 mg of the 2:1 theaflavin:epicatechin combination. All experimental plates were incubated at 37°C for 24 h, after which the zones of inhibition were recorded (mm). Six-fold replication of all experimental plates was performed and the results were provided as mean values  $\pm$  SE. Disc diffusion assays were also repeated after stock solutions had been stored for 7 days at 4°C to assess whether the stability of the compounds might affect the reproducibility of tests conducted using stored discs.

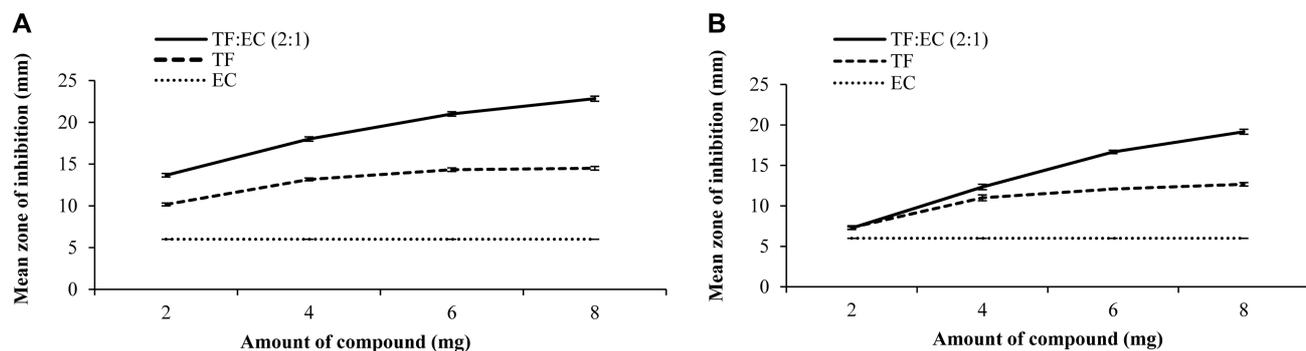
## Results and Discussion

Epicatechin alone had little activity against *C. albicans* NCTC 3255 or NCTC 3179 ( $>2,048$  µg/ml), but both strains were inhibited by theaflavin (1,024 µg/ml) (Table 1). This confirms previous findings that theaflavin alone has antifungal activity but with much lower MIC values than those previously reported [18]. Analysis of the results from the tests using 2:1 combinations of theaflavin and epicatechin revealed an even greater antifungal effect than that observed using solutions containing theaflavin alone, with MICs of

**Table 1.** Minimum inhibitory concentration and fractional inhibitory concentration indexes of theaflavin, epicatechin, and theaflavin:epicatechin (2:1) combinations against isolates of *Candida albicans*.

<i>C. albicans</i> isolate number	Minimum inhibitory concentration (µg/ml)			
	TF	EC	TF + EC	FICIs
NCTC 3255	1,024 (0)	>2,048 (0)	256 (0)	0.313
NCTC 3179	1,024 (0)	>2,048 (0)	128 (0)	0.188

MIC = minimum inhibitory concentration, FICIs = fractional inhibitory concentration indexes, TF = theaflavin, EC = epicatechin.



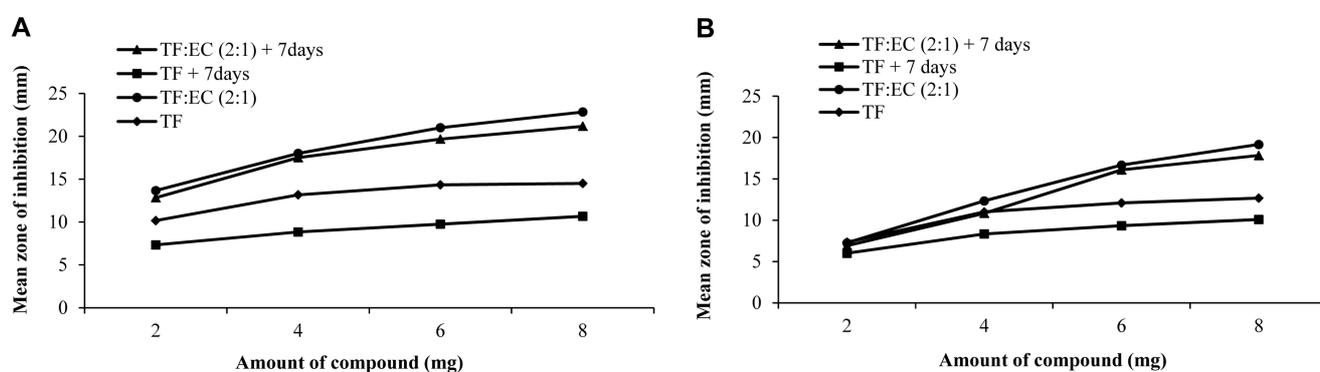
**Fig. 1.** Antifungal effects of theaflavin (TF), epicatechin (EC), and synergy between TF and EC against *C. albicans* NCTC 3255 (A) and NCTC 3179 (B).

Error bars are the standard error of the mean of three measurements. Zones include a 6 mm disc diameter.

256 and 128  $\mu\text{g}/\text{ml}$  for *C. albicans* isolates NCTC 3255 and 3179, respectively. The noticeable and significant increase in antifungal activity suggests that synergy exists between the two polyphenols, confirmed by the FICIs of  $<0.5$  (Table 1).

In the disc diffusion assays, zones of inhibition ranged from 10.2 mm ( $\pm 0.41$ ) at 2 mg/disc to 14.5 mm ( $\pm 0.54$ ) at 8 mg/disc against isolate NCTC 3255, and between 7.3 mm ( $\pm 0.52$ ) at 2 mg/disc and 12.6 mm ( $\pm 0.52$ ) at 8 mg/disc against isolate NCTC 3179. A significant difference in activity was observed in assays using theaflavin and epicatechin (2:1) in combination (Fig. 1). Combinations of theaflavin and epicatechin (2:1) tested against *C. albicans* isolate NCTC 3255 produced zones of inhibition ranging between 13.6 mm ( $\pm 0.52$ ) at 2 mg/disc and 22.8 mm ( $\pm 0.75$ ) at 8 mg/disc. Zones of inhibition produced by (2:1) combinations of theaflavin:epicatechin against isolate NCTC 3179 range between 7.3 mm ( $\pm 0.42$ ) at 2 mg/disc and 19.2 mm at 8 mg/disc.

Disc diffusion assays conducted with stock solutions stored for 7 days at 4°C showed noticeable differences compared with those performed with stock solutions used immediately after preparation (Fig. 2). After 7 days, epicatechin remained inactive against both strains of *C. albicans*, but the antifungal action of theaflavin was also seen to be reduced. Discs containing 8 mg of TF generated mean zones of inhibition of 10.7 mm ( $\pm 0.41$ ) and 10.1 mm ( $\pm 0.20$ ) for isolates NCTC 3255 and NCTC 3179, respectively, equivalent to a 26% and 20% reduction in activity, respectively. This could result from oxidation of the theaflavin, reducing the amount of the biologically active polyphenol in the formulation. Stored discs containing the theaflavin:epicatechin (2:1) combinations also showed reduced antifungal activity but to a much lesser extent, with a 7% and 7.1% reduction in activity, respectively. The reason behind the lower reduction in activity could be a protective effect of theaflavin by epicatechin, with epicatechin acting as an antioxidant and



**Fig. 2.** Antifungal effects of theaflavin (TF) and synergy between TF and epicatechin (EC) against *C. albicans* NCTC 3255 (A) and NCTC 3179 (B) when used immediately and after storage at 4°C for 7 days.

Mean results of three measurements. Zones include a 6 mm disc diameter.

reducing the rate at which theaflavin is oxidized, thus preserving a greater concentration of “active” theaflavin. This interpretation would be compatible with the results from a previous work, where the antioxidant ascorbic acid was found to prolong the activity lifespan of the polyphenol epigallocatechin gallate [8]. Further studies using HPLC analysis should be undertaken to calculate optimum storage times for theaflavin:epicatechin solutions. With the addition of another antioxidant, such as ascorbic acid, the shelf life of theaflavin and theaflavin:epicatechin combinations might be further increased.

Although the mechanisms responsible for polyphenol synergy are not fully understood, possible explanations include protection of theaflavin by epicatechin, similar to previous observations [8] in which ascorbic acid was shown to enhance the antibacterial activity of epigallocatechin gallate. Although the mechanism for the antimicrobial activity of polyphenols is unknown, previous studies have shown that theaflavins and catechins cause extensive damage to the cell wall of *C. albicans* [18]. The addition of epicatechin to theaflavin might intensify this pro-oxidant effect by further weakening the cell wall prior to any interaction with theaflavin, thereby reducing the concentration of theaflavin required for antifungal activity.

In conclusion, the results of this preliminary study highlight the synergy between theaflavin and epicatechin, when used against *C. albicans*, which could be harnessed to improve their antifungal activity. Any potential use of theaflavin:epicatechin combinations as a systemic treatment will need further investigation, given the relatively high MICs observed *in vitro* and the poor bioavailability of polyphenols [11]. However, a topical preparation could be useful as a treatment for skin or mucosal *C. albicans* infections, capitalizing on both the antifungal and antioxidant properties [10], which may be advantageous in promoting wound healing. Future research should look into the mechanisms behind the intrinsic, synergistic antimicrobial and antioxidant properties of these natural compounds

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