

Original Paper

Short-Term Antifungal Treatments of Caprylic Acid with Carvacrol or Thymol Induce Synergistic 6-Log Reduction of Pathogenic *Candida albicans* by Cell Membrane Disruption and Efflux Pump Inhibition

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Key Words

Candida albicans • Caprylic acid • Essential oil component • Synergistic antifungal activity • Membrane disruption • Efflux pump

Abstract

Background/Aims: Although naturally-derived antifungals have been investigated for their ability to inactivate *Candida albicans*, which is a major cause of candidiasis, they have shown a less than 3 log reduction in *C. albicans* or required treatment times of longer than 3 h. Thus, the naturally-derived antifungals used in previous studies could not substantially eradicate *C. albicans* within a short period of time. **Methods:** To improve the fungicidal effects of naturally-derived antifungals against *C. albicans* within short time periods, we developed composites showing antifungal synergism using caprylic acid (CA), carvacrol (CAR) and thymol (THM) for 1–10 min at 22/37°C. Using flow cytometry, we examined the mode of action for the synergism of these compounds on membrane integrity and efflux pump activity. **Results:** Whereas the maximum reduction by individual treatments was 0.6 log CFU/ml, CA + CAR/THM (all 1.5 mM) eliminated all pathogens (> 6.8 log reduction) after 1 min at 37°C and after 10 min at 22°C. The flow cytometry results showed that exposure to CA damaged the membranes in 15.7–36.5% of cells and inhibited efflux pumps in 15.4–31.3% of cells. Treatments with CAR/THM slightly affected cell membranes (in 1.8–6.9% of cells) but damaged efflux pumps in 14.4–29.6% of cells. However, the combined treatments clearly disrupted membranes (> 83.1% of cells) and pumps (> 95.0% of cells). The mechanism of this synergism may involve membrane damage by CA, which facilitates the entry of antifungals into the cytoplasm, and the inhibition of efflux pumps by CA, CAR or THM, causing their accumulation within cells and, leading to cell death.

Conclusion: Antifungal composites (CA + CAR/THM) showing synergism (i.e., an additional 6 log reduction) within minutes at room/body temperature can be used to treat candidiasis and improve the microbiological safety of facilities contaminated with fungi as a novel alternative to synthetic antifungals.

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Introduction

Fungal infections have emerged as a major public health issue worldwide, and there are growing numbers of immunocompromised patients (1). Of all fungal diseases, candidiasis is one of the most common opportunistic and nosocomial infections, which are most commonly caused by *Candida albicans*, accounting for 42.5% of cases of candidiasis (2). *C. albicans* is commonly found in the intestinal tract, mouth, throat, vagina or nails (3). More than 75% of women have vulvovaginal candidiasis once in their lifetime, and 30–50% of healthy individuals carry this pathogen as a part of their normal oral microflora (4). Furthermore, *C. albicans* is often disseminated from mucosal lesions, gastrointestinal colonization or unsterile medical instruments, such as catheters or dentures (5). The spread of *C. albicans* can lead to invasive candidiasis, which is the fourth most common bloodstream infection, afflicting 250,000 patients and causing more than 50,000 deaths worldwide every year (6). Even with antifungal therapy, the mortality of invasive candidiasis is greater than 40%. Thus, it is important to inactivate *C. albicans* (i.e., destroy the biological activities of *C. albicans*) before the pathogen spreads to other body areas.

A variety of intervention methods designed to inactivate *C. albicans* have been investigated. *C. albicans* is enveloped within a plasma membrane and cell wall, protecting it from harsh environments and playing important roles in pathogenesis (7, 8). Thus, the primary targets for controlling *C. albicans* are potentially related to the membrane and cell wall (7). Traditionally, antibiotics have been used in the prevention or treatment of candidiasis. Azoles are mostly used to inactivate *C. albicans*, and are drugs that work by inhibiting the biosynthetic pathway of ergosterol, which is a component of the cell membrane (9). However, the prolonged use of antibiotics results in drug-resistant *C. albicans* (10). Clinical resistance to antibiotics can cause gastrointestinal or endocrinologic disorders and oxidative drug metabolism in the liver (11, 12). A significant mechanism of antibiotic resistance is the overexpression of efflux pumps, which actively export drugs out of cells (13, 14). Thus, the inhibition of drug efflux pumps is considered to be an important method for inactivating fungi (10, 12, 15). Due to the undesirable side effects of antibiotic overuse, the development of new antifungal compounds as alternatives to antibiotics is necessary, with naturally-derived antimicrobials representing preferred sources for new pharmaceutical products (16).

However, the naturally-derived antimicrobial compounds used in previous studies cannot sufficiently eradicate *C. albicans* within a short time. For example, some studies showed slight inhibitory effects (< 3 log CFU/ml reduction) of naturally-derived antifungal agents (e.g., lauric acid, eugenol, thymol, and carvacrol) after treatments of more than 3 h (9, 17, 18). Thus, to increase the efficacy of naturally-derived antifungals, hurdle technologies using naturally-derived agents have been developed, and a number of studies have focused on the combined effects of antifungal compounds. The use of several antifungals in combination is expected to achieve a synergistic effect and require lower quantities of the antifungals (19). Our previous study demonstrated that combination technologies using various types of naturally-derived antimicrobial compounds (e.g., medium-chain fatty acids, essential oil compounds, organic acids, citrus fruit extracts, etc.) showed marked antibacterial effects against a variety of foodborne pathogenic bacteria (e.g., *Escherichia coli* O157:H7, *Salmonella* Typhimurium, *Cronobacter* spp., etc.) compared to treatments with individual compounds. Following these studies, we attempted to determine which naturally-derived agents optimally inactivate *C. albicans*. However, pathogenic fungi have greater resistance

to environmental stresses than bacteria due to having nuclear membranes and greater cell sizes, making the elimination of fungi potentially more difficult than that for bacteria (20).

Among the many naturally-derived compounds that have been studied, caprylic acid (CA), carvacrol (CAR) and thymol (THM) were further investigated in this study based on their antifungal synergism within minutes, low required concentrations, pH value and nontoxicity toward humans. CA is a medium-chain fatty acid (MCFA) that is naturally present in coconut oil and mammalian breast milk and is used to improve microbiological safety in the food, health care and medical industries (9, 19). CAR and THM are essential oil components (EOs) extracted from various plant materials (seeds, flowers, buds, herbs, woods and roots) and have been used as naturally-derived antimicrobial compounds and preservatives (10, 21, 22). However, no studies have examined the fungicidal activity and mechanism of action of the combined treatment of CA, CAR and THM against *C. albicans*.

The aim of this study was to develop a novel complex of CA and EOs (CAR or THM) with synergistic fungicidal activity after short exposure times against *C. albicans* at room temperature (22°C) and body temperature (37°C). Moreover, to elucidate the mode of action underlying the antifungal effects of the combined treatment, the accumulation of antifungals in the fungal cytoplasm due to increased membrane permeability and the inhibition of efflux pump activity was analyzed.

Materials and Methods

Preparation of fungal cell suspensions

Three strains of *C. albicans* (ATCC 10231, 18804, and 11006) were obtained from the American Type Culture Collection and the Food Microbiology Culture Collection of Korea University (Seoul, Korea). Stock cultures were stored at -80°C in sabouraud dextrose broth (SDB; Difco, Becton Dickinson, Sparks, MD, USA) containing 20% glycerol and were sub-cultured on a monthly basis. Each *C. albicans* strain was separately cultured at 30°C for 24 h in SDB. To prepare the three-strains cocktail, all culture suspensions were centrifuged at 1,800 × g for 15 min (CentraCL2; International Equipment Company, Needham Heights, MA, USA). After discarding the supernatants, the three pellets were washed twice with 0.85% saline, resuspended in 0.85% sterile saline and combined in a sterile 50-ml centrifuge tube (Becton Dickinson, Franklin Lakes, NJ, USA) to yield the prepared cell suspension (approximately 9 log CFU/ml).

Antifungal efficacy tests of CA combined with CAR or THM

CA, CAR and THM were purchased from Sigma Chemical Co. (St. Louis, MO, USA). CA stock solutions (100 and 150 mM) and CAR or THM stock solutions (50, 100 and 150 mM) were prepared in 98% ethanol (EtOH) and used within a week after their preparation.

For the individual CA, CAR and THM treatments, a 0.1 ml aliquot of CA, CAR or THM stock solution was added to 9.8 ml of sterile 0.85% saline in a sterile glass tube (final CA concentrations: 1 or 1.5 mM; final CAR or THM concentrations: 0.5, 1, or 1.5 mM). For combination treatments of CA with CAR or THM, the two CA solutions (1 and 1.5 mM) were individually combined with each EO solution (CAR or THM; 0.5, 1 and 1.5 mM). Aliquots (0.1 ml) of the CA and EO stock solutions were added to 9.7 ml of sterile 0.85% saline (0.1 ml of CA + 0.1 ml of the EOs + 9.7 ml of saline, total volume 9.9 ml). The tubes containing each antimicrobial solution were then equilibrated to 22 or 37°C at 100 rpm in a shaking water bath (VS-1205SW1, Vision Scientific Co., Ltd., Daejeon, Korea) prior to the addition of 0.1 ml of *C. albicans* suspension to yield an initial cell density of approximately 7 log CFU/ml. All tested fungi were exposed to treatment solutions for 1, 5 or 10 min at 22 or 37°C in a shaking water bath (100 rpm). The fungal suspension was also added to 9.9 ml of 0.85% saline to estimate the initial concentration of the inoculum and to a 2% EtOH solution as a solvent control. All experiments were repeated six times.

Microbiological analysis

Candida samples (1 ml) that had been treated with the antimicrobial agents were diluted 10-fold in 0.85% sterile saline (9 ml), and 0.1 ml aliquots were spread onto duplicate sabouraud dextrose agar (SDA) plates. To lower the detection limit, 0.2 ml of undiluted sample were spread onto five plates (a total of 1 ml;

the detection limit was 1 CFU/ml). The plates were then incubated at 35°C for 48 h to allow surviving *C. albicans* cells to form colonies, which were subsequently enumerated.

The recovery of injured cells was evaluated in samples treated with antifungal composites, which showed complete inactivation of the inoculated *C. albicans*. Treated samples (1 ml) were inoculated in 30 ml of SDB. After incubating at 30°C for 24 h, the enriched samples were streaked onto SDA plates. The recovery was reported as positive or negative according to the formation of colonies after the SDA plates were incubated at 35°C for 48 h. The experiments were repeated six times.

Stability test for antifungal efficacy of CA combined with CAR or THM

To examine the stability of the antifungal effects of CA combined with CAR or THM, long-term storage tests of the composites were performed. The antifungal effects of the CA + CAR or THM solutions stored for 14, 30, and 60 days at room temperature (22°C) were measured. Prepared *C. albicans* cell suspensions were added to each stored solution, and the microbiological analysis was performed as described in sections 2.2–2.3. In addition, the pH values of CA + CAR or THM stored for 0, 14, 30 and 60 days were measured using a pH/ion meter (S220 SevenCompact pH/Ion, Mettler-Toledo, Greifensee, Switzerland) at 22 or 37°C. The pH meter was calibrated with commercially available standard buffer solutions at pH 7.0, 4.0 and 2.0 (Mettler Toledo). The experiments were performed in triplicate.

Evaluation of pH effect on antifungal efficacy of combined treatments

To evaluate the net effect of the pH value on the synergistic antifungal activity of the antifungal composites, *C. albicans* cells were exposed to hydrochloric acid (HCl) solutions. For the same pH values of 1.5 mM CA (pH 4.0 at 22 and 37°C), 0.1 ml-aliqouts of 0.18 mM HCl solution were added to 9.8 ml of sterile 0.85% saline in sterile glass tubes (0.0018 mM final HCl concentration, pH 4.0). Additionally, 0.1 ml of 0.18 mM HCl and 0.1 ml of CAR or THM stock solution were added to 9.7 ml of sterile 0.85% saline solution to prepare solutions with the same pH values as those of the 1.5 mM CA + 1.5 mM CAR or THM samples (pH 4.0 at 22 and 37°C). Individual and combined antifungal efficacy tests were performed as described above. The pH of each solution was determined as described in section 2.4. The experiments were performed in triplicate.

Analysis of membrane damage by flow cytometry via propidium iodide staining

Flow cytometry was performed to identify effects of CA, CAR or THM alone, or in combination (CA + CAR or THM), and of HCl solutions (pH 4.0) alone or in combination with 1.5 mM CAR or THM (pH 4.0), on the integrity of cell membranes. Propidium iodide (PI) molecular probe reagent (Invitrogen, Thermo Fisher Scientific Inc., Grand Island, NY, USA), a nucleic acid-binding fluorescent probe, was used to evaluate the effect of antifungals on cell membrane lesions. *C. albicans* suspensions (approximately 7 log CFU/ml) of untreated cells (living cells), treated cells, or dead cells (after treatment with 100% EtOH for 10 min) were prepared in sterile phosphate-buffered saline (PBS; pH 7.4) (23). Immediately after treatment, the cells were diluted 4-fold in sterile PBS to prevent further fungicidal activity. Residual chemicals were removed by three washes with centrifugation at $18,341 \times g$ for 3 min at 4°C (Smart R17 Plus, Hanil Scientific Inc., Seoul, Korea). The diluted *C. albicans* cells were then reconcentrated (4-fold). All target cells were stained with 1.0 µg/ml of PI at 35°C for 30 min in the dark. Subsequently, the stained cells were washed three times with sterile PBS and used for flow cytometry analysis.

Flow cytometry was performed with a FACSCalibur flow cytometer (Becton Dickinson Biosciences, San Jose, CA, USA) using a blue argon laser (15 mW) with an excitation wavelength of 488 nm. Data were analyzed using BD CellQuest Pro (Becton Dickinson Biosciences). Orange fluorescence emitted by PI was detected at ≤ 585 nm (FL2 channel). The signals were detected as logarithmic signals from a photodiode detector with a forward scatter voltage setting of E00, with data acquisition including 30,000 events at a flow rate of 12 µl/min. The results were depicted as histograms, where the M1 region was defined as live and dead cells, and the percentage of fungal cells in the M1 region was calculated using BD CellQuest Pro (Becton Dickinson, San Jose, CA). The experiments were performed in triplicate.

Analysis of efflux pump activity by flow cytometry with Nile red staining

The efflux pump activities of *C. albicans* cells treated with CA, CAR or THM alone; CA + CAR or THM; HCl solution (pH 4.0) alone; and HCl + CAR or THM (pH 4.0) were examined via flow cytometry by measuring the

efflux of Nile red dye (Sigma-Aldrich, St. Louis, MO, USA) (24). Nile red is a specific substrate for *C. albicans* efflux pumps. Samples were prepared as described in section 2.6, except that PBS containing 2% glucose and 7 μ M Nile red was used instead of PBS and 1.0 μ g/ml PI. Flow cytometry was performed as described in section 2.6, except that the red fluorescence emitted by Nile red was detected at \leq 670 nm (FL3 channel). The experiments were performed in triplicate.

Statistical analysis

Duplicate average plate counts from six repeated experiments were converted to log number of CFU/ml. All experimental fungal reduction data were examined by analysis of variance (ANOVA) using SAS version 9.4 (SAS Institute, Cary, NC, USA). Significant differences were determined using Tukey's multiple comparisons tests ($P < 0.05$).

Results

Antifungal efficacy of CA combined with CAR or THM at 22°C

Fig. 1 shows the fungicidal effects of CA (1 and 1.5 mM) and the two EOs (0.5, 1, and 1.5 mM) alone or in combinations of CA with CAR or THM (1 + 0.5, 1 + 1, 1 + 1.5, 1.5 + 0.5, 1.5 + 1, and 1.5 + 1.5 mM) for 1, 5 and 10 min at 22°C. Control treatments (2% EtOH) showed no antifungal effects in any of the experiments (< 0.1 log reduction; $P > 0.05$). The maximum reduction by individual treatments was only 0.2 log CFU/ml with 1.5 mM CA for 1–10 min. For CA with 0.5 mM CAR or THM, fungicidal effects were not observed even when the treatment time and CA concentration were increased. Treatments of 1 mM CA combined with 1 mM CAR or THM also showed negligible reductions in *C. albicans* populations (< 0.6 log reduction). The antifungal effects of 1 mM CA + 1.5 mM CAR or THM were augmented by increasing treatment time. In these assays, a maximum reduction of 4.0 log CFU/

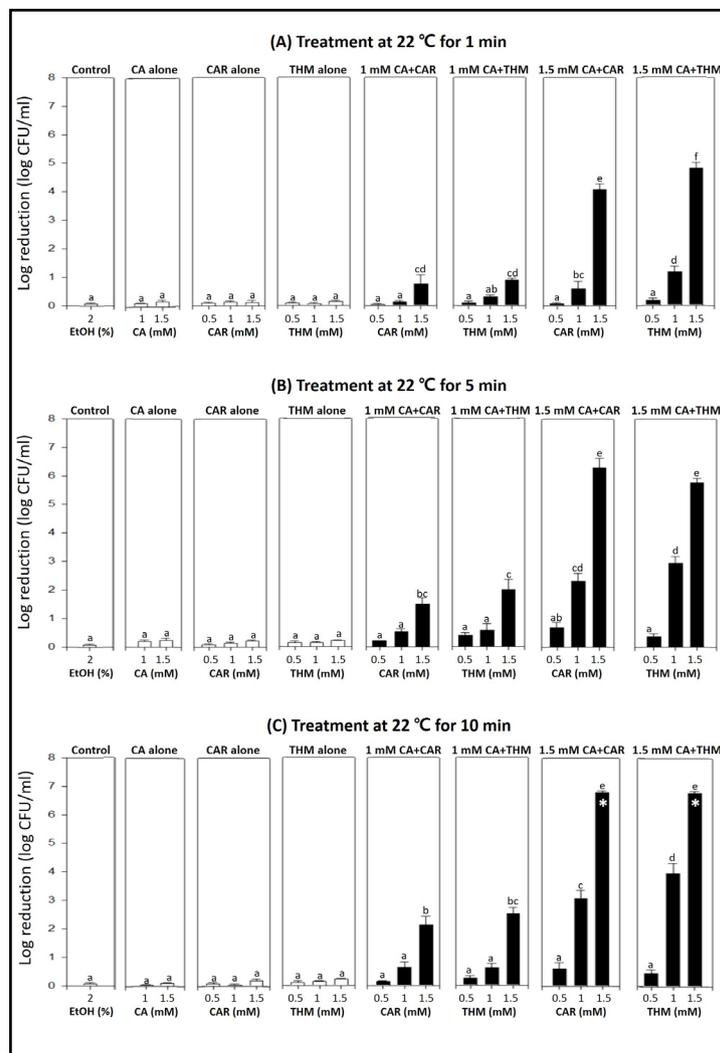


Fig. 1. Antifungal effects of 2% EtOH, CA (1 and 1.5 mM) alone, EOs (CAR or THM; 0.5, 1 and 1.5 mM) alone, or the combinations of CA and EOs after treatment for 1 min (A), 5 min (B), 10 min (C) at 22°C. Data bars show the mean \pm standard deviation ($n = 6$). Data indicated by different superscripts (a-f) are significantly different ($P < 0.05$). *Complete elimination in enrichment tests ($n = 6$).

ml was observed after treatment for 10 min, although the treatments for 1 min with the composites resulted in less than 1.2 log reductions. The combined treatments of 1.5 mM CA + 1.5 mM CAR or THM showed highly enhanced fungicidal effects, where after 10 min at 22°C, the composite treatments reduced the fungal population to undetectable levels (initial population: 6.8 log CFU/ml; detection limit: 1 CFU/ml). Indeed, the treatments resulted in more than 6.5 log CFU/ml reductions in *C. albicans* cells, although the sum of the antifungal effects achieved by individual treatments was only a 0.3 log reduction in the fungal population. The elimination of inoculated *C. albicans* cells was confirmed by enrichment tests.

Antifungal efficacy of CA combined with CAR or THM at 37°C

The antifungal effects of CA (1 and 1.5 mM), CAR or THM (0.5, 1, and 1.5 mM) alone and in combined treatments on target fungi are shown in Fig. 2. When fungal suspensions were treated with CA, CAR and THM alone for 10 min, negligible reductions in *C. albicans* suspension were observed (< 0.6 log reduction). However, the combined treatments with

CA and CAR or THM showed synergistic antifungal effects.

Antifungal synergism was enhanced by specific treatment times, temperatures, and concentrations of CA, CAR and THM. When *C. albicans* suspensions were exposed to composites of 1.5 mM CA + 0.5 mM CAR or THM and 1 mM CA + 1 mM CAR or THM at 37°C for 10 min, 2.4–3.3 log reductions in fungal population were observed, although the treatments at 22°C resulted in less than 0.6 log reduction. The combined treatments of 1 mM CA + 1.5 mM CAR or THM and 1.5 mM CA + 1 mM CAR or THM showed 3.0–3.7 log reductions in *C. albicans* cells after 1 min and 5.1 log reduction after 10 min. Indeed, the antifungal effects of the composites at 37°C were higher than those observed at 22°C. When 1.5 mM CA was used in combination with 1.5 mM CAR or THM at any time point, all fungal cells were entirely eradicated (> 6.9 log CFU/ml reduction, detection limit: 1 CFU/ml), and no cells were detected after further enrichment in SDB, whereas treatments of the composites at 22°C for

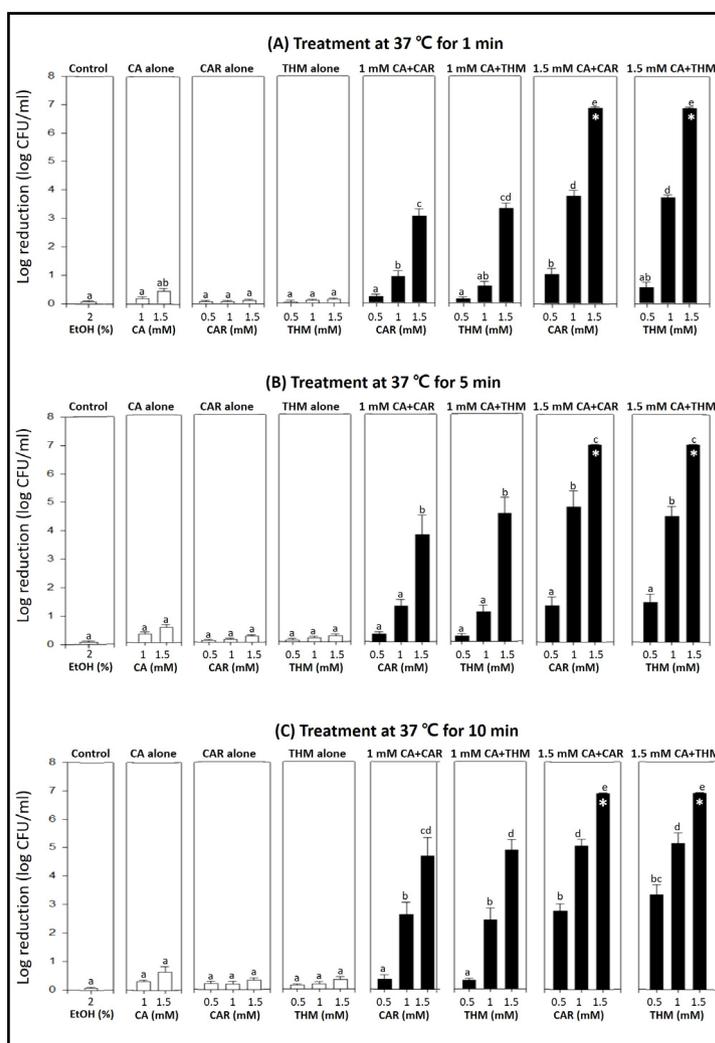


Fig. 2. Antifungal effects of 2% EtOH, CA (1 and 1.5 mM) alone, EOs (CAR or THM; 0.5, 1 and 1.5 mM) alone, or the combinations of CA and EOs after treatment for 1 min (A), 5 min (B), 10 min (C) at 37°C. Data bars show the mean ± standard deviation (n = 6). Data indicated by different superscripts (a-f) are significantly different (P < 0.05). *Complete elimination in enrichment tests (n = 6).

Table 1. Stability for antifungal effects of CA (1.5 mM) combined with CAR or THM (1.5 mM) stored for 14, 30, and 60 days. ^aData represent mean \pm standard deviation (n = 3). ^bThe detection limit was 1 CFU/ml. ND, not detected

Combined treatment	Temperature (°C)	Time (min)	Microbial reduction after storage period for 14, 30 and 60 days at 22°C (log CFU/ml) ^a		
			14 days	30 days	60 days
1.5 mM CA + 1.5 mM CAR	22	10	6.9 \pm 0.0 (ND) ^b	5.1 \pm 0.1	5.1 \pm 0.1
	37	1	6.9 \pm 0.0 (ND)	6.9 \pm 0.0 (ND)	5.7 \pm 0.1
	37	5	6.9 \pm 0.0 (ND)	6.9 \pm 0.0 (ND)	6.9 \pm 0.0 (ND)
	37	10	6.9 \pm 0.0 (ND)	6.9 \pm 0.0 (ND)	6.8 \pm 0.0 (ND)
1.5 mM CA + 1.5 mM THM	22	10	6.9 \pm 0.0 (ND)	5.2 \pm 0.0	5.1 \pm 0.1
	37	1	6.9 \pm 0.0 (ND)	6.9 \pm 0.0 (ND)	5.9 \pm 0.2
	37	5	6.9 \pm 0.0 (ND)	6.9 \pm 0.0 (ND)	6.9 \pm 0.0 (ND)
	37	10	6.9 \pm 0.0 (ND)	6.9 \pm 0.0 (ND)	6.9 \pm 0.0 (ND)

1–5 min did not reduce the *C. albicans* populations to undetectable levels. The 1.5 mM CA + 1.5 mM CAR or THM treatments showed additional 6.0–6.3 log reductions within 1–10 min, whereas the sums of log reductions by individual treatment ranged from 0.6–0.9 log reductions in *C. albicans* cells within 1–10 min.

Stability of the antifungal efficacy of CA combined with CAR or THM

Because the 1.5 mM CA + 1.5 mM CAR or THM treatments for 10 min at 22°C and for 1–10 min at 37°C showed the greatest synergistic antifungal effects (Fig. 1 and 2), the composites were stored for 14, 30, and 60 days to determine the stability of their antifungal efficacy and assess any changes in pH. After storage for 0–60 days, the pH values were maintained at 4.0 (data not shown). The 1.5 mM CA and 1.5 mM CAR or THM composites stored for 14 days eradicated all fungal cells in an inoculum (> 6.9 log CFU/ml reduction) treated for 10 min at 22°C and for 1–10 min at 37°C (Table 1). When fungal suspensions were treated at 22°C for 10 min with the composites stored for 30 and 60 days, 5.1–5.2 log CFU/ml reductions of fungal populations were observed. Similarly, treatments with CA + CAR or THM stored for 60 days at 37°C for 1 min reduced the population of *C. albicans* by 5.7–5.9 log CFU/ml. Treatments for 10 min at 37°C with the composites showed the highest fungicidal activity observed (> 6.8 log reduction).

Effects of CA, CAR or THM alone or in combination on cell membrane integrity

Based on the treatment conditions that showed the most synergistic effects at 22 or 37°C in the previous experiments, *C. albicans* was treated with 1.5 mM CA, 1.5 mM CAR or THM or a combination thereof (1.5 mM CA + 1.5 mM CAR or THM) at 22°C for 10 min and at 37°C for 1–10 min. The cells were subsequently stained with PI to analyze the membrane integrity of the treated cells by flow cytometry (Fig. 3). The proportions of damaged cells exposed to 1.5 mM CA alone were higher than those exposed to 1.5 mM CAR or THM under the same treatment times and temperatures. For example, the percentages of permeabilized fungal cells induced by 1.5 mM CA were 15.7% and 18.7%–36.5% after treatment for 10 min at 22°C and for 1–10 min at 37°C, respectively (Fig. 3a). However, Fig. 3b and 3c show that treatments with 1.5 mM CAR and THM alone had lower effects on cells when tested at each treatment condition (10 min at 22°C, 1.8–2.0%; 1–10 min at 37°C, 3.0–7.6%). Additionally, the percentages of permeabilized cells treated by each antifungal agent alone tended to increase with treatment temperature. When *C. albicans* cells were treated with the same compound for the same exposure duration (10 min), permeabilization was higher at 37°C [36.5% after 10 min treatment at 37°C with 1.5 mM CA (Fig. 3a); 6.9–7.6% after 10 min treatment at 37°C with 1.5 mM CAR or THM (Fig. 3b and 3c)] than that at 22°C [15.7% after 10 min treatment at 22°C with 1.5 mM CA (Fig. 3a); 1.8–2.0% after 10 min treatment at 22°C with 1.5 mM CAR or THM (Fig. 3b and 3c)]. The combined treatments resulted in 95.0–99.8% of fungal cells being permeabilized when treated at 22°C for 10 min and at 37°C for 1–10 min (Fig. 3d and 3e).

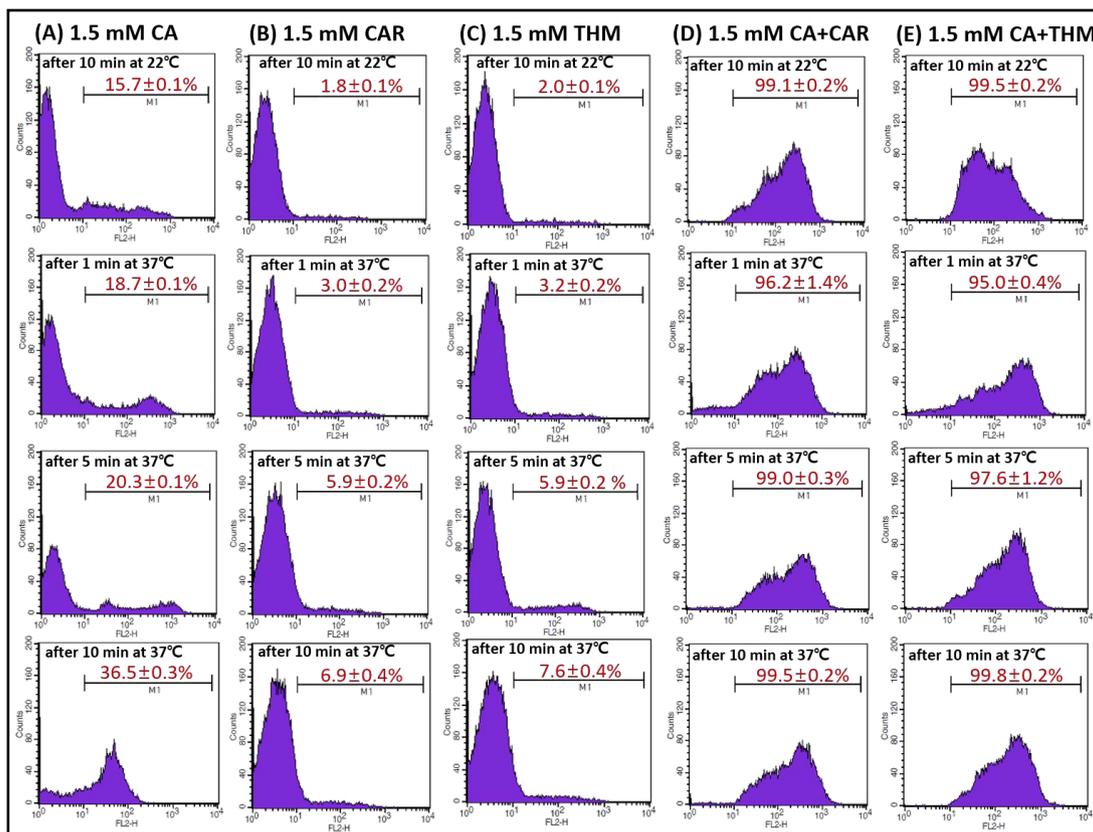


Fig. 3. Flow cytometry analysis with PI staining showing the membrane damage of *C. albicans* cells treated with 1.5 mM CA alone (A), 1.5 mM CAR alone (B), 1.5 mM THM alone (C), 1.5 mM CA + 1.5 mM CAR (D), and 1.5 mM CA + 1.5 mM THM (E) for 10 min 22°C or for 1–10 min for 37°C. The percentage of cells in M1 shows the mean \pm standard deviation ($n = 3$).

Effects of CA, CAR or THM alone or in combination on efflux pump integrity

According to the treatment conditions that resulted in the most marked fungicidal effects at 22 or 37°C, efflux pump activities after treatments with 1.5 mM CA, CAR or THM or their combinations thereof for 10 min at 22°C or for 1–10 min at 37°C were evaluated using flow cytometry by measuring the efflux of Nile red dye (Fig. 4). The results showed that individual treatments of 1.5 mM CA, CAR or THM considerably inhibited efflux pump activity [15.4–31.3% treated with 1.5 mM CA, (Fig. 4a); 14.4–29.6% of cells with 1.5 mM CAR or THM (Fig. 4b and 4c)]. Nile red accumulated at higher levels in *C. albicans* cells treated with CA, CAR or THM alone at 37°C for 10 min (25.8–31.3%) than those treated at 22°C (14.4–15.4%). Indeed, efflux pump function gradually decreased with increasing temperature. For the combined treatments, efflux pump activity was completely inhibited in 83.1–99.1% of cells (Fig. 4d and 4e).

Examination of the effect of pH on the antifungal efficacy of combined treatments

Because CA is a weak acid, the pH values of combined solutions were determined relative to CA (1.5 mM CA has a pH of 4.0). To measure the effects of pH on the synergistic fungicidal effects, fungal cells were exposed to 0.0018 mM HCl (pH 4.0) and 0.0018 mM HCl + 1.5 mM EOs (pH 4.0) for 10 min at 22 and 37°C (Table 2). Individual treatments of 0.0018 mM HCl at 22 and 37°C did not show any fungicidal effects (< 0.1 log CFU/ml reduction), while 1.5 mM CA alone caused a less than 0.4 log reduction in a *C. albicans* suspension. The combined treatments of 0.0018 mM HCl with 1.5 mM CAR or THM at 22 and 37°C resulted in less than 0.5 log reductions, whereas the combined treatments of 1.5 mM CA + 1.5 mM CAR or THM for 10 min at 22 and 37°C inactivated all fungal cells (> 6.7 log CFU/ml reductions).

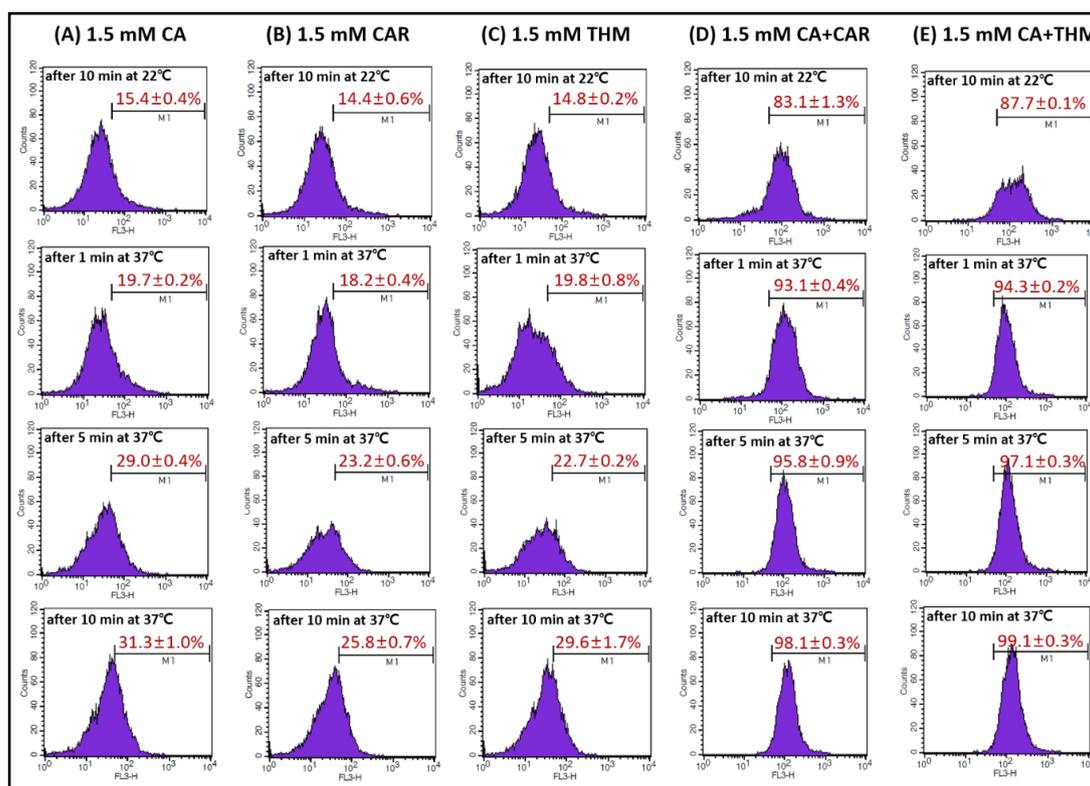


Fig. 4. Flow cytometry analysis with Nile red staining showing the efflux pump activity of *C. albicans* cells treated with 1.5 mM CA alone (A), 1.5 mM CAR alone (B), 1.5 mM THM alone (C), 1.5 mM CA + 1.5 mM CAR (D), and 1.5 mM CA + 1.5 mM THM (E) for 10 min at 22°C or for 1–10 min for 37°C. The percentage of cells in M1 shows the mean ± standard deviation (n = 3).

Damages to *C. albicans* membranes and efflux pumps induced by treatments with HCl (pH 4.0) alone or in combination with 1.5 mM CAR or THM (pH 4.0) for 10 min at 22°C and 37°C were also examined (Fig. 5 and 6). Unlike the combined treatments with 1.5 mM CA + 1.5 mM CAR or THM (Fig. 3 and 4), exposure to HCl + CAR or THM showed negligibly damaged membranes and efflux pumps. The percentage of *C. albicans* cells with membranes that were damaged by HCl alone and by HCl + CAR or THM was only 2.1–4.2% (Fig. 5c and 5d) and 2.7–6.6% (Fig. 5e to h), respectively. The HCl treatments only slightly inhibited the efflux pump activity of *C. albicans* [1.4–3.7% of cells (Fig. 6c and 6d)], whereas the combined treatments of HCl and CAR or THM blocked transporter pump activity in 13.1–28.0% of cells (Fig. 6e to h).

Table 2. Antifungal effects of the individual or combined treatment of HCl, CA, CAR and THM after 10 min at 22°C and 37°C. ^aData represent mean ± standard deviation (n = 3). Data indicated by different superscripts (a, b) in the same column are significantly different (P < 0.05). ^bThe detection limit was 1 CFU/ml. ND, not detected

Treatment	Reduction (log CFU/ml) ^a	
	22°C	37°C
Control (2% EtOH)	0.1 ± 0.0 ^a	0.1 ± 0.0 ^a
Individual treatment		
0.0018 mM HCl (pH 4.0)	0.1 ± 0.0 ^a	0.1 ± 0.1 ^a
1.5 mM CA (pH 4.0)	0.4 ± 0.1 ^a	0.3 ± 0.1 ^a
1.5 mM CAR (pH 6.2)	0.1 ± 0.0 ^a	0.2 ± 0.0 ^a
1.5 mM THM (pH 6.2)	0.4 ± 0.1 ^a	0.2 ± 0.0 ^a
Combined treatment		
0.0018 mM HCl + 1.5 mM CAR (pH 4.0)	0.2 ± 0.1 ^a	0.5 ± 0.1 ^a
0.0018 mM HCl + 1.5 mM THM (pH 4.0)	0.4 ± 0.1 ^a	0.4 ± 0.1 ^a
1.5 mM CA + 1.5 mM CAR (pH 4.0)	6.8 ± 0.1 ^b (ND)	6.7 ± 0.0 ^b (ND)
1.5 mM CA + 1.5 mM THM (pH 4.0)	6.8 ± 0.1 ^b (ND)	6.7 ± 0.0 ^b (ND)

Fig. 5. Flow cytometry analysis with PI staining showing the membrane damage of *C. albicans* cells that were untreated (A) or treated with 100% EtOH for 10 min to make dead cells (B), HCl (pH 4.0) alone for 10 min at 22°C (C) or 37°C (D), HCl + 1.5 mM CAR (pH 4.0) and HCl + 1.5 mM THM (pH 4.0) for 10 min at 22°C (E and F) and for 10 min at 37°C (G and H). The percentage of cells in M1 shows the mean ± standard deviation (n = 3).

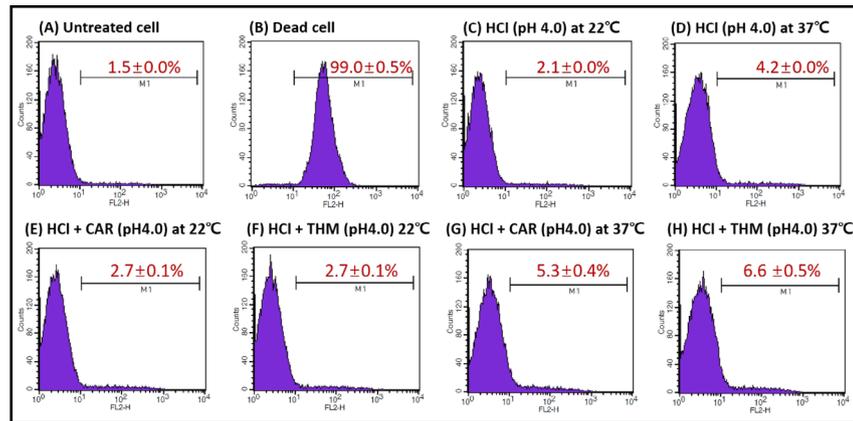
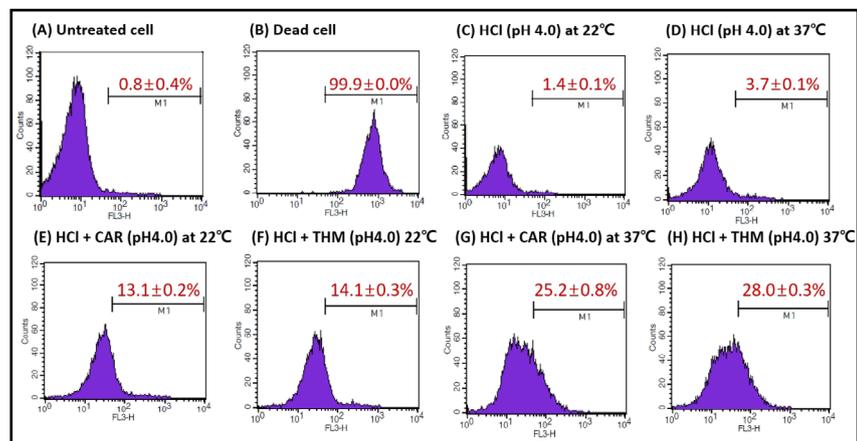


Fig. 6. Flow cytometry analysis with Nile red staining showing the efflux pump activity of *C. albicans* cells that were untreated (A) or treated with 100% EtOH for 10 min to make dead cells (B), HCl (pH 4.0) alone for 10 min at 22°C (C) or 37°C (D), HCl + 1.5 mM CAR (pH 4.0) and HCl + 1.5 mM THM (pH 4.0) for 10 min at 22°C (E and F) and for 10 min at 37°C (G and H). The percentage of cells in M1 shows the mean ± standard deviation (n = 3).



Discussion

The results of the present study showed that combined treatments of CA and CAR or THM had highly enhanced fungicidal effects against *C. albicans*. The antifungal activity of the complexes was strengthened as the treatment concentrations (CA, 1–1.5 mM; CAR or THM, 0.5–1.5 mM), treatment time (1–10 min) and temperature (22 and 37°C) increased. The optimal treatment conditions that resulted in the complete eradication of *C. albicans* (> 6.8 log reduction) included 1.5 mM CA + 1.5 mM CAR or THM for 10 min at 22°C or for 1 min at 37°C. Indeed, with this combinatorial approach, only low concentrations of antifungals were necessary for the elimination of the pathogen within minutes. Comparatively, the maximum sum of the log reductions achieved by individual treatments was less than a 0.9 log reduction. Given that *C. albicans* was not inactivated by CA, CAR or THM alone, their combination can improve the usefulness of naturally-derived agents in controlling *C. albicans* infections in contaminated environments.

Previous studies have reported the potential of CA, CAR and THM in controlling *C. albicans* but showed limited fungicidal effects. For example, previous studies on the antifungal effects of CA showed negligible anti-*Candida* effects, despite the use of high CA concentrations (> 10 mM) and long exposure times (> 10 min) at 30–37°C (17, 25). Studies on the antifungal effects

of 2.0–8.0 mM CAR and THM observed less than 3 log reductions of *C. albicans* after 3–48 h at 35–37°C (9, 26, 27). These results do not meet industrial standards due to low antifungal activities and requisite long exposure times. To improve the efficiency of naturally-derived antifungals, combination treatments with other antimicrobial agents (e.g., naturally-derived components and antibiotics) (28, 29) or physical methods (e.g., ultraviolet irradiation) (30) have been developed. However, the combined treatments assayed in previous studies showed insufficient fungicidal effects against *C. albicans* despite long exposure times (24–48 h). In contrast, our study demonstrated that the working concentrations of antifungals (CA, CAR and THM) can be reduced when using combined treatments. Indeed, the highest individual concentration in these combined treatments was only 0.024% (1.5 mM) of CA. Moreover, these composites were stable after 60 days of storage, as high antifungal effects and low pH values of CA + CAR or THM were maintained. The antifungals assayed in this study (CA, CAR and THM) are representative antioxidants that inhibit oxidation by scavenging free reactive alkyl, alkoxy, or peroxy radicals, leading to an elevated stability of the treated product. Thus, these findings suggested that the antioxidant activities of CA, CAR and THM contributed to their stability in water-based solutions to maintain their high bioactivity (31–34). The weak antifungal effects, long treatment times, and high concentrations of chemical agents observed in previous studies were overcome in this study using combined treatments at both 22 and 37°C.

The total molecular masses of CA and CAR or THM are 144 and 150 Da, respectively. Because these compounds are able to pass through the *Candida* cell wall, which is permeable to solutes smaller than 600 Da (35, 36), their mechanism of antifungal activity involves membrane lesions (9, 25, 37–39). However, the mode of action underlying the synergistic fungicidal activity of CA+ CAR or THM has yet to be elucidated. CA comprises a carbon chain with single bonds and a terminal carboxyl group (-COOH) and is likely to incorporate into the fungal cell membrane as a nonionic surfactant (19, 25, 37, 40). Previous studies have indicated that CA alters membrane permeability and induces fungal cell death by diffusing into and creating permanent pores in the cell membranes. CAR and THM are structural isomers and lipophilic monoterpene phenols (21, 22). The primary antimicrobial mechanisms of CAR or THM are known to involve altering the fluidity of membranes or inhibiting efflux pumps (9, 12, 41). Based on the results of previous studies, we hypothesized that the highly enhanced antifungal activity of the combined treatments of CA and CAR or THM may be due to the disruption of cell membrane integrity and the blocking of efflux pumps reducing the intrinsic resistance of *C. albicans* to CA + CAR or THM.

In this study, we showed that *C. albicans* cells treated with CA alone exhibited increased cell membranes damage than the assayed EOs alone via PI staining and flow cytometry. Conventional plating methods showed that the individual CA, CAR and THM treatments did not have any fungicidal activity (Fig. 1 and 2). However, flow cytometry analysis showed that exposure to CA alone caused considerable cell membrane damage (in 15.7–36.5% of cells), whereas individual treatments with CAR or THM showed negligible membrane disruption (in 1.8–7.6% of cells). These results suggested that individual treatment with 1.5 mM antifungals caused membrane damage, but this damage could be recovered. In addition to membrane damage analysis, efflux pump activity inhibition by treatment with antifungals was analyzed via flow cytometry using Nile red dye. Treatment with CA alone considerably increased membrane permeability and substantially inhibited efflux pump activity (in 15.4–31.3% of cells). However, the intensity of Nile red in *C. albicans* cells exposed to CAR or THM was higher than that observed for untreated cells (in 14.4–29.6% of cells). These results contrasted with those obtained via flow cytometry and PI staining, which showed that fungal cell membrane integrity was slightly affected (in 1.8–7.6% of cells). When *C. albicans* cells were exposed to 1.5 mM CA combined with 1.5 mM CAR or THM, the cell membranes and efflux pumps were completely disrupted [in 95.0–99.8% of cells (Fig. 3d and 3e), in 83.1–99.1% of cells (Fig. 4d and 4e)], indicating that irreversible membrane damage and cytoplasm leakage occurred. The flow cytometric analyses of the combined treatments were consistent with the findings obtained using the conventional plating method, indicating the elimination of all fungi after

treatments for 10 min at 22°C and for 1–10 min at 37°C. Based on these results, we propose the following mechanism for the synergistic fungicidal activity of CA + CAR or THM: (i) the permeability of the cell membrane was increased primarily by CA, (ii) the activity of drug efflux pumps was also reduced by CAR or THM as well as CA, (iii) the increased membrane permeability facilitated the influx of other antifungal compounds (CA, CAR or THM) into the cytoplasm, and (iv) the direct entry of the antifungals and inhibition of their efflux resulted in organelle destruction (e.g., nucleus, mitochondria, Golgi body, etc.), leading to cell death.

We also observed that the treatment temperature affected the antifungal activity of the developed composites, which altered the integrity of the cell membrane and efflux pump activity. Blicher et al. described a relationship between the heat capacity and permeation rate of membranes comprising dipalmitoyl phosphatidylcholine (DPPC) and dipalmitoyl phosphatidylglycerol (DPPG) (42). The study showed that temperatures of 36.5–41.4°C increased the permeability of the DPPC/DPPG membrane, whereas temperatures below 36.5°C did not affect permeability. Blicher et al. suggested that high temperatures facilitate pore formation that is sufficient for permeability to ions, water, and large molecules (e.g., proteins, peptides, glucose, sugars, etc.) (43, 44). These studies support that the combined treatment at 37°C inactivated *C. albicans* and damaged membranes, including efflux pumps, for short duration treatments (1 min) compared with treatments at 22°C (10 min).

Because low pH plays an important role in hurdle technology, the acidity of CA, which is a weak acid (pH 4.0 at 1.5 mM), would be one of the factors responsible for the synergistic antifungal effects. However, as shown in Table 2, the composites of HCl + CAR or THM adjusted to the pH value of CA + CAR or THM (pH 4.0) did not show fungicidal activity (< 0.5 log reduction) as well as HCl alone (pH 4.0). In addition, in Fig. 3 and 5, the damage to cell membranes induced by the composites of HCl and CAR or THM (in 2.7–6.6% of cells) was less than that induced by the composites of CA and CAR or THM (in 95.0–99.8% of cells). In Fig. 4 and 6, the inhibition of efflux pump activity by HCl + CAR or THM (in 13.1–28.0% of cells) was primarily due to the antifungal activity of CAR or THM, whereas the individual treatment of 1.5 mM CAR or THM blocked efflux pump activity in 14.4–29.6% of cells. Lambert et al. reported that charged ions, such as hydrogen ions, does not easily interact with or return across the cell membrane (45). Based on previous studies and the results of this study, the antifungal activity of CA was considered to involve changes in membrane permeability and diffusion of nondissociated acid molecules through the membrane into the cytoplasm rather than due to pH reduction. Because the efflux pump activity was inhibited by CA, the diffused nondissociated molecules, dissociated hydrogen ions, and anions are concentrated within the cell, resulting in the disruption of cytoplasmic organelles. Therefore, the contribution of the fungicidal activity of CA to the antifungal synergism with CAR or THM was attributed to the direct damage of cell membranes and efflux pumps rather than the acidity of CA.

The strengths of the antifungal complex developed in this study are as follows: (i) all of the antifungal compounds used are naturally-derived materials and not synthetic agents, and this strength is important since many consumers who have negative images of chemical compounds tend to prefer using naturally-derived agents rather than synthetic ones due to concern over the perceived toxicity of chemicals (21); (ii) the developed complex is safe for consumption because CA, CAR and THM are generally recognized as safe by the United States Food and Drug Administrations; and (iii) the efficiency of each antifungal compound to inactivate *C. albicans* is increased through their combined use at low concentrations with very short exposure times. Fungi such as *C. albicans* are generally more resilient than bacteria because they are approximately 25–50 times larger and have more complex organelles, such as nuclear membranes, which can act as an additional barrier to antimicrobials (46, 47). To the best of our knowledge, this study is the first to show that combination technology with only naturally-derived antifungals can completely eliminate *C. albicans* with highly resistant characteristics within minutes based on marked synergism.

The antifungal complex showing synergism at 22°C (room temperature) can be utilized in health care centers, pharmaceutical and medical industries for the prevention of cutaneous candidiasis. Conventional chemical agents used in cleansers or antiseptic cause skin irritation,

such as dryness, and are harmful to infants and children, who have less developed immune systems than those of adults (48-51). The antifungal complex can be utilized as novel surface-sanitizer in facilities such as animal farms where the occurrence of fungal contamination is higher with livestock and commercial sanitizers, such as sodium hypochlorite, have little antimicrobial effects (52, 53). Additionally, the combined treatment with naturally-derived agents presented in this study showing synergistic antifungal effects at 37°C (i.e., body temperature) can be used as active ingredients in anti-candidiasis pharmaceuticals or dietary supplements and coating agents of medical devices (e.g., catheters and dentures) susceptible to *C. albicans* contamination (54-57). However, the required concentration to achieve sufficient antifungal effects *in vivo* is higher than that required in fungal suspension due to the presence of human tissues or the host immune status (21). Thus, the next logical step is to optimize the combined treatment of these compounds required to induce the strongest antifungal effect and to examine their effects on clinical specimens or surfaces.

Conclusion

The results of the present study revealed that the optimized combinations of CA and CAR or THM at low concentrations had synergistic fungicidal effects within short durations against *C. albicans*. These combinations of naturally-derived agents may be promising alternatives to synthetic compounds used to control pathogenic fungi. We analyzed the changes in membrane integrity and efflux pump activity of treated *C. albicans* cells using flow cytometry. The synergistic mechanism may involve the increased entry of the antifungals into the cells due to the disruption of the fungal membrane, primarily caused by CA, and by inhibition of the efflux of antifungals by CA, CAR or THM. These novel findings can potentially lead to the development of a new antifungal approach to cope with human fungal infection and environmental fungal contamination.

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Yoon Seol Bae wrote the manuscript and Prof Min Suk Rhee assisted in experimental design, data interpretation and critical revision of the manuscript. All authors read and approved the final version.

Disclosure Statement

The authors have no conflicts of interest to declare.

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