RESEARCH ARTICLE

Synergistic anticandidal activity of xanthorrhizol in combination with ketoconazole or amphotericin B

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Abstract

Candida species are responsible for the fourth most common nosocominal bloodstream infection. Xanthorrhizol, a sesquiterpene compound isolated from *Curcuma xanthorrhiza* Roxb. has been reported to have anticandidal activity. The aim of this study is to investigate the synergistic anticandidal effect of xanthorrhizol in combination with ketoconazole or amphotericin B against *Candida albicans*, *Candida glabrata*, *Candida guilliermondii*, *Candida krusei*, *Candida parapsilosis*, and *Candida tropicalis*. Mostly, xanthorrhizol in combination with ketoconazole or amphotericin B exhibited the synergistic anticandidal effects against all species of *Candida* tested. In combination with xanthorrhizol, the concentration of ketoconazole or amphotericin B for inhibiting the growth of the tested *Candida* species could be reduced by $\geq$50%. Time–kill curves showed that 1/2 minimum inhibitory concentration (MIC) dose of xanthorrhizol, amphotericin B, or ketoconazole alone against each of the six *Candida* species did not inhibit the growth of all *Candida* species tested. However, 1/2 MIC dose of xanthorrhizol in combination with 1/2 MIC dose of ketoconazole or 1/2 MIC dose of amphotericin B exhibited growth inhibition of all *Candida* species tested and reduced viable cells by several logs within 4 h. These results support the potential use of xanthorrhizol as an anticandidal agent, and it can be used complementarily with other conventional antifungal agents.

Introduction

Candida species are a significant cause of infection and the population at risk includes cancer patients and transplant recipients treated with immunosuppressive drugs. Amphotericin B and drugs of the azole group, such as ketoconazole, have been extensively used in the treatment of candidal infections. Unfortunately, the widespread use of these antifungals has led to the emergence of drug resistance in several common pathogenic fungi (Alexander & Perfect, 1997; Cuenca-Estrella *et al.*, 2005). In addition, fungi that were previously regarded as saprobes are now occurring on the human host, with some of these fungal species having innate resistance to the presently available antifungal drugs (Vartivarian *et al.*, 1993; Graybill, 1996). The growth of some fungal saprobes on human skin has become a problem. They are capable of infecting damage or devitalized skin, primarily causing skin disease (Dierauf & Gulland, 2001). Moreover, the used therapeutics against mycoses are often toxic and may result in risky drug–drug interactions (Shin & Pyun, 2004).

The increasing resistance to antifungal compounds and the reduced number of available drugs led us to search for therapeutic alternatives among plant essential oils, because they are promising sources for new natural antifungal drugs, even though they have relatively mild effects against human pathogenic fungi compared with commercial synthetic antifungal drugs (Devkatte *et al.*, 2005; Cavelario *et al.*, 2006; Pauli, 2006). In our previous reports, xanthorrhizol isolated from the rhizome of Java turmeric (*Curcuma xanthorrhiza*) showed significant antifungal activity. Xanthorrhizol possessess antimycotic activity against opportunistic

filamentous fungi such as *Aspergillus flavus*, *Aspergillus fumigatus*, *Aspergillus niger*, *Fusarium oxysporum*, *Rhizopus oryzae*, and *Trichophyton mentagrophytes* (Rukayadi & Hwang, 2007b). Xanthorrhizol also has anti-*Malassezia* activity against *Malassezia furfur* and *Malassezia pachydermatis* (Rukayadi & Hwang, 2007c). Interestingly, xanthorrhizol has broad anticandidal activity against *Candida albicans* and non-*C. albicans* species, such as *Candida glabrata*, *Candida guilliermondii*, *Candida krusei*, *Candida parapsilosis*, and *Candida tropicalis* (Rukayadi *et al.*, 2006).

The potential enhancement of antifungal effects of natural products administrated in combination with synthetic drugs has been investigated previously (Carson *et al.*, 2002; Shin & Pyun, 2004). The objective of the current study is to evaluate the *in vitro* synergistic anticandidal effect of xanthorrhizol in combination with amphotericin B or ketoconazole against six *Candida* species, namely *C. albicans*, *C. glabrata*, *C. guilliermondii*, *C. krusei*, *C. parapsilosis*, and *C. tropicalis* using checkerboard titer tests and time–kill curve analysis.

Materials and methods

Candida strains and inoculum preparation

The six reference *Candida* species (*C. albicans* ATCC 22972, *C. glabrata* ATCC 90525, *C. guilliermondii* ATCC 20216, *C. krusei* ATCC 6258, *C. parapsilosis* ATCC 7330, and *C. tropicalis* ATCC 42678) were obtained from the American Type Culture Collection (Rockville, MD). Clinical *Candida* isolates were obtained from The Research Institute of Bacterial Resistance, College of Medicine, Yonsei University, Korea. The clinical isolates were isolated from body fluid, blood, urine, sputum, and genitals of the patients. The species were identified by conventional methods or using Vitek YBC cards (bioMérieux, Marcy l'Etoile, France) according to the manufacturer's instruction. The *Candida* strains were cultured in Sabouraud dextrose broth (SDB) or on Sabouraud dextrose agar (SDA) (Difco, Spark, MD) for 48 h at 35–37 °C. A standardized inoculum for each isolate of *Candida* was prepared as follows: the *Candida* species were propagated in SDB at 35–37 °C for 24 h with 200 r.p.m. agitation. One milliliter of a 24-h-old culture in SDB was centrifuged (3900 ***g*** at 4 °C for 1 min), and the pellets were washed twice with 1 mL of physiological saline. Sterile physiological saline was added to give a McFarland turbidity of 0.5 at 530 nm, corresponding to 5×10^6 CFU mL^{-1} [Clinical Laboratory Standards Institute (CLSI), 2002].

Antifungal agents

Xanthorrhizol (Fig. 1) was isolated as a pure form from the ethylacetate fraction of the methanol extract of *C. xanthorrhiza* according to the method of Hwang *et al.* (2000). Briefly, the rhizomes (100 g) were ground and extracted with 75% MeOH (v/v; 400 mL), and further fractionations were carried out consecutively with ethyl acetate (4.8 g), *n*-butanol (1.7 g) and water (1.1 g). Xanthorrhizol (0.2 g) was isolated from the ethyl acetate fraction using a silica gel column chromatography (Merck; 70–230 mesh; 5 × 43 cm; *n*-hexane/ethyl acetate, 10 : 1). Xanthorrhizol was identified by direct comparison of the ^{1}H-nuclear magnetic resonance (NMR), ^{13}C-NMR, and electrospray ionization-mass spectral results with the published data (Itokawa *et al.*, 1985).

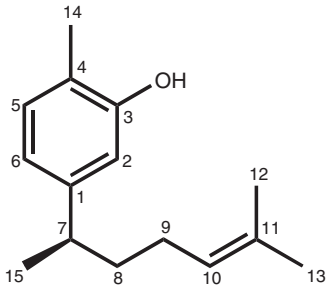

Fig. 1. Structure of xanthorrhizol.

Amphotericin B and ketoconazole were purchased from Sigma Chemical Co. (St. Louis, MO). Amphotericin B, ketoconazole, and xanthorrhizol were dissolved in 5% dimethyl sulfoxide (DMSO) (Samchun, Pyeongtaek City, Korea) to obtain 512 μg mL^{-1} stock solutions (CLSI, 2002). DMSO at 5% was found not to kill the *Candida* strains.

Determination of minimum inhibitory concentration (MIC)

MICs of amphotericin B, ketoconazole, and xanthorrhizol on the six *Candida* species were evaluated using the method described in the guidelines of CLSI M27-A2 (2002). Briefly, MICs were determined for all species with the adjusted inoculum suspension of 5×10^6 CFU mL^{-1} by diluting 1 : 100 with 3-(*N*-morpholino)propanesulfonic acid (MOPS)-buffered Roswell Park Memorial Institute (RPMI) 1640 medium to obtain the final inoculum concentration of 5×10^4 CFU mL^{-1}. Individual antifungal agents were diluted with 1 : 10 in MOPS-buffered RPMI 1640 medium containing 5×10^4 CFU mL^{-1} inoculum, yielding an initial inoculum of 4.5×10^3 CFU mL^{-1}. A 200-μL aliquot of each suspension was placed in 96-well round-bottom microtitration plates. The plates were incubated at 35–37 °C and endpoints were read visually after 48 h. The anticandidal activity of 5% (v/v) DMSO was also studied on a separate microtiter plate alongside the assay. Controls containing anticandidal agents in broth and broth with inocula were also included. The MICs were interpreted as the lowest concentration of antifungal agent that totally inhibited the growth of the tested *Candida*

strains compared with the growth control, after the preset incubation time. The experiments were performed four times with four replicate wells for each experiment.

Human cell toxicity determination

Human hepatoma (HepG2) cells were used to evaluate the toxicity effect of xanthorrhizol with and without ketoconazole and amphotericin B using the method described by Zhang *et al.* (2007), with a slight modification. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, a tetrazole) assay (Sigma-Aldrich, St. Louis, MO) was used to determine the relative cell viability, according to the manufacturer's instruction.

Briefly, cells HepG2 were cultured in RPMI medium 1640, supplemented with 10% fetal bovine serum (Thermo Scientific, Lafayette, CO), 100 U mL^{-1} streptomycin, 100 mg mL^{-1} penicillin, 4 mM L-glutamine, 1% nonessential amino acids, and 1 mM sodium pyruvate. Cells were maintained at 37 °C and 5% CO_2 in a humidified incubator. From the top to the bottom of 96-well microtiter plate there were a series of twofold dilutions of the compounds: xanthorrhizol alone in columns 1 and 2, ketoconazole alone in columns 3 and 4, amphotericin B alone in columns 5 and 6, xanthorrhizol in combination with ketoconazole in columns 7 and 8, and xanthorrhizol in combination with amphotericin B in columns 9 and 10. Wells in columns 11 and 12 were treated as controls (cells not exposed to anticandidal agent). The same amount of HepG2 cells was seeded in each of the selected wells and incubated for 24 h at 37 °C CO_2 incubator with a humidified chamber. A 30-μL solution of MTT (5 mg mL^{-1} in phosphate-buffered saline) was added in each of the selected wells and incubated for 2 h at 37 °C. Relative cell viability was determined at 595 nm with a tunable microplate reader ($VERSA_{MAX}$, Sunnyvale, CA). The experiments were performed four times with duplicate wells for each experiment.

Checkerboard titer tests

The anticandidal activity of xanthorrhizol in combination with amphotericin B or ketoconazole was assessed in a suspension assay by the checkerboard method (Shin & Lim, 2004). In brief, serial double dilutions of the anticandidal compounds were prepared 0.125–512 μg mL^{-1}. Fifty microlitres of each amphotericin B or ketoconazole dilution was added to the rows of a 96-well microtitre plate in diminishing concentrations and 50 μL of the xanthorrhizol was also added to the columns in diminishing concentrations. A 100-μL suspension of *Candida* strains adjusted to 5×10^5 CFU mL^{-1} was added to each well and cultured at 35–37 °C for 48 h. The MIC of both anticandidal compounds in combination was determined as described above. To assess the synergistic or antagonistic activity of anticandidal combinations, the fractional inhibitory concentrations (FICs) and FIC index (FICI) were determined. FICs were calculated by dividing the MIC of the combination of xanthorrhizol and amphotericin B or ketoconazole by the MIC of xanthorrhizol, amphotericin B, or ketoconazole alone. The FICI was calculated by adding both FICs. The FICI was interpreted as synergistic when it was ≤ 0.5, as antagonistic when > 4.0, and any value in between as indifferent (Karpanen *et al.*, 2008). The experiment was repeated four times with four replicates per experiment.

Time–kill curves analysis

Time–kill analyses were performed in standard MOPS-buffered RPMI 1640 (CLSI, 2002). Before the tests were performed, the *Candida* species were subcultured at least twice and grown for 24 h at 35–37 °C on SDA plates. Each concentration of amphotericin B, ketoconazole, and xanthorrhizol was diluted with 1 : 10 in MOPS-buffered RPMI 1640 medium containing 5×10^6 CFU mL^{-1} of *Candida* strains. This procedure yielded an initial inoculum of 4.5×10^6 CFU mL^{-1}. Each *Candida* strain experiment was conducted in six groups of culture tubes with the final concentrations as follows: (1) control (without an antifungal agent), (2) 1/2 MIC dose of xanthorrhizol, (3) 1/2 MIC dose of ketoconazole or amphotericin B, (4) MIC dose of xanthorrhizol, (5) MIC dose of ketoconazole or amphotericin B, and (6) 1/2 MIC dose of xanthorrhizol combined with 1/2 MIC dose of ketoconazole or amphotericin B. A 1/2 MIC is defined as the half of the lowest concentration of an anticandidal agent that will inhibit the visible growth of a *Candida* isolate after 48 h of incubation. Cultures (5-mL final volume) were incubated at 35–37 °C with agitation (200 r.p.m.). At predetermined time points (0, 15, 30 min, 1, 2, 3 and 4 h), a 100-μL aliquot was removed and transferred to Eppendorf tubes, centrifuged (3900 ***g*** at 4 °C for 1 min) and rinsed twice with 0.9 mL of sterile distilled water to obtain antifungal agent-free cells. Pellets were suspended in 100 μL of sterile distillated water and serially diluted. An appropriate volume (100, 40, or 20 μL), depending on the dilution and the concentration of antifungal agent, were spread onto SDA plates and incubated at 35–37 °C for 48 h or more (until the colonies were seen on the plates) to determine the numbers of CFU mL^{-1}. The experiment was repeated four times with four replicates per experiment.

Statistical analysis

Data were expressed as mean ($n = 4 \times 4$). Statistical significance was determined by ANOVA (SPSS 11.0 for Windows). A *P*-value of < 0.05 was considered as statistically significant.

Results

MICs of amphotericin B, ketoconazole, and xanthorrhizol against six *Candida* species are summarized in Table 1. To evaluate xanthorrhizol with and without ketoconazole and amphotericin B that may be toxic to human cells, we used HepG2 cells as a surrogate system to mimic potential therapeutic side effects in human body. The top wells, columns 1 and 2, containing 16 μg mL^{-1} xanthorrhizol showed ≥98% cell viability compared with that of the control (Fig. 2). A fixed concentration of 4 μg mL^{-1} ketoconazole or amphotericin B in combination with series dilution of xanthorhhizol (columns 7 and 8, 9 and 10, respectively) did not kill HepG2 cells; cell viability in each of the tested wells was ≥95% compared with that of the control (Fig. 2). These results indicated that xanthorrhizol with and without ketoconazole and amphotericin B is not toxic to human cells. The checkerboard titer assay indicates significant combined effects between xanthorrhizol and ketoconazole or amphotericin B as shown in Tables 2 and 3. The MICs of xanthorrhizol, ketoconazole, and amphotericin B alone against six *Candida* species were significantly higher than MICs in combination (xanthorrhizol–ketoconazole or xanthorrhizol–amphotericin B). FICI of xanthorrhizol in combination with ketoconazole against *C. albicans*, *C. glabrata*, *C. guilliermondii*, *C. parapsilosis*, *C. krusei*, and *C. tropicalis* were 0.25–0.625, 0.156–0.75, 0.25–0.375, 0.375, 0.25–0.75, and 0.375, respectively (Table 2). Mostly, xanthorrhizol in combination with amphotericin B has a synergistic anticandidal effect against *C. albicans*, *C. guilliermondii*, *C. krusei*, and *C. tropicalis* with FICI values of 0.25–0.75, 0.25–0.5, 0.25–0.5, 0.25, 0.25–1.0, and 0.375, respectively. These data indicate that xanthorrhizol in combination with ketoconazole or amphotericin B exhibited the synergistic anticandidal effects against all species of *Candida* tested. In this study, antagonism was not observed for any of combinations.

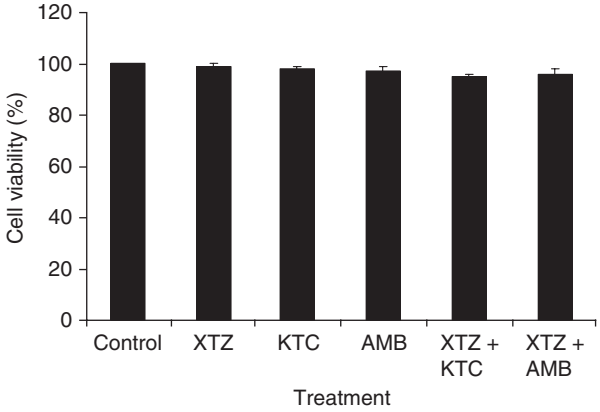

Fig. 2. Nontoxicity effect of xanthorrhizol (XTZ, 16 μg mL^{-1}) with and without ketoconazole (KTC, 4 μg mL^{-1}), and amphotericin B (AMB, 4 μg mL^{-1}) to human hepatoma (HepG2) cells. Cells were cultured without anticandidal as a control. Data are expressed as % control and each column represents the mean ± SD of four independent experiments.

Table 1. *In vitro* anticandidal activity of xanthorrhizol, amphotericin B, and ketoconazole

	MIC (μg mL^{-1})		
Candida species	Xanthorrhizol	Amphotericin B	Ketoconazole
C. albicans ATCC 22972	4.0	1.0	1.0
C. albicans (clinical)			
B2756	4.0	0.5	1.0
B4381	4.0	0.5	0.5
C372	4.0	0.5	1.0
C372	2.0	0.5	1.0
P173	2.0	0.5	0.5
G61	4.0	1.0	0.5
C. glabrata ATCC 90525	8.0	1.0	1.0
C. glabrata (clinical)			
B869	1.0	0.5	0.5
B1226	4.0	0.5	0.5
B240	4.0	1.0	1.0
B532	2.0	0.5	0.5
B115	4.0	0.5	1.0
C. guilliermondii ATCC 20216	4.0	1.0	0.5
C. guilliermondii (clinical)			
C25	4.0	0.5	1.0
B5050	4.0	0.5	1.0
C. krusei ATCC 6258	4.0	1.0	1.0
C. parapsilosis	16.0	1.0	0.5
ATCC 7330			
C. parapsilosis (clinical)			
B1153	8.0	1.0	1.0
C276	16.0	1.0	0.5
B3421	8.0	0.5	1.0
B1089	8.0	0.5	0.5
B392	8.0	0.5	1.0
C. tropicalis ATCC 42678	2.0	0.5	1.0

The results of time–kill curve analysis are presented in Figs 3 and 4. Figure 3 shows the effect of 1/2 MIC dose of xanthorrhizol or 1/2 MIC dose of ketoconazole alone or in combination against six *Candida* species. The effects of 1/2 MIC dose of xanthorrhizol or 1/2 MIC dose of amphotericin B alone or in combination against six *Candida* species are shown in Fig. 4. A 1/2 MIC dose of xanthorrhizol, amphotericin B, or ketoconazole alone against each of the six *Candida* species did not significantly affect the growth of all *Candida* species tested. However, 1/2 MIC dose of xanthorrhizol in combination with 1/2 MIC dose of ketoconazole to all *Candida* species tested and 1/2 MIC dose of xanthorrhizol in combination with 1/2 MIC dose of amphotericin B to *C. albicans*, *C. guilliermondii*, *C. krusei*, and *C. tropicalis* significantly exhibited fungicidal activity. The fungicidal activity of 1/2 MIC dose of xanthorrhizol in combination

Table 2. Checkerboard assay of xanthorrhizol and ketoconazole against *Candida* species

		MIC ($\mu g\ mL^{-1}$) of each antifungal agent				
Candida species	Antifungal agent	Alone	Combination	FIC ($\mu g\ mL^{-1}$)	FICI	Outcome
C. albicans ATCC 22972	Xanthorrhizol	4.0	1.0	0.25	0.375	Synergistic
	Ketoconazole	1.0	0.125	0.125		
C. albicans (clinical)						
B2756	Xanthorrhizol	4.0	0.5	0.2	0.45	Synergistic
	Ketoconazole	1.0	0.25	0.25		
B4381	Xanthorrhizol	4.0	0.5	0.2	0.45	Synergistic
	Ketoconazole	0.5	0.125	0.25		
C372	Xanthorrhizol	4.0	1.0	0.25	0.5	Synergistic
	Ketoconazole	1.0	0.25	0.25		
C372	Xanthorrhizol	2.0	0.25	0.125	0.25	Synergistic
	Ketoconazole	1.0	0.125	0.125		
P173	Xanthorrhizol	2.0	0.25	0.125	0.625	Indifference
	Ketoconazole	0.5	0.125	0.5		
G61	Xanthorrhizol	4.0	1.0	0.25	0.5	Synergistic
	Ketoconazole	0.5	0.125	0.25		
C. glabrata ATCC 90525	Xanthorrhizol	8.0	2.0	0.25	0.375	Synergistic
	Ketoconazole	1.0	0.125	0.125		
C. glabrata (clinical)						
B869	Xanthorrhizol	1.0	0.125	0.125	0.375	Synergistic
	Ketoconazole	0.5	0.125	0.25		
B1226	Xanthorrhizol	4.0	0.5	0.125	0.375	Synergistic
	Ketoconazole	0.5	0.125	0.25		
B240	Xanthorrhizol	4.0	1.0	0.25	0.75	Indifference
	Ketoconazole	1.0	0.5	0.5		
B532	Xanthorrhizol	2.0	0.5	0.25	0.5	Synergistic
	Ketoconazole	0.5	0.125	0.25		
B115	Xanthorrhizol	4.0	0.125	0.031	0.156	Synergistic
	Ketoconazole	1.0	0.125	0.125		
C. guilliermondii ATCC 20216	Xanthorrhizol	4.0	0.5	0.125	0.375	Synergistic
	Ketoconazole	0.5	0.125	0.25		
C. guilliermondii (clinical)						
C25	Xanthorrhizol	4.0	1.0	0.25	0.375	Synergistic
	Ketoconazole	1.0	0.125	0.125		
B5050	Xanthorrhizol	4.0	0.5	0.125	0.25	Synergistic
	Ketoconazole	1.0	0.125	0.125		
C. krusei	Xanthorrhizol	4.0	1.0	0.25	0.375	Synergistic
ATCC 6258	Ketoconazole	1.0	0.125	0.125		
C. parapsilosis	Xanthorrhizol	16.0	4.0	0.25	0.5	Synergistic
ATCC 7330	Ketoconazole	0.5	0.125	0.25		
C. parapsilosis (clinical)						
B1153	Xanthorrhizol	8.0	4.0	0.5	0.625	Indifference
	Ketoconazole	1.0	0.125	0.125		
C276	Xanthorrhizol	16.0	4.0	0.25	0.5	Synergistic
	Ketoconazole	0.5	0.125	0.25		
B3421	Xanthorrhizol	8.0	2.0	0.125	0.25	Synergistic
	Ketoconazole	1.0	0.125	0.125		
B1089	Xanthorrhizol	8.0	4.0	0.5	0.75	Indifference
	Ketoconazole	0.5	0.125	0.25		
B392	Xanthorrhizol	8.0	2.0	0.125	0.25	Synergistic
	Ketoconazole	1.0	0.125	0.125		
C. tropicalis	Xanthorrhizol	2.0	0.5	0.25	0.375	Synergistic
ATCC 42687	Ketoconazole	1.0	0.125	0.125		

FIC, fractional inhibitory concentration (FIC = MIC combination/MIC alone); FICI = FIC of xanthorrhizol + FIC of ketoconazole.

Table 3. Checkerboard assay of xanthorrhizol and amphotericin B against *Candida* species

		MIC ($\mu g\,mL^{-1}$) of each antifungal agent				
Candida species	Antifungal agent	Alone	Combination	FIC ($\mu g\,mL^{-1}$)	FICI	Outcome
C. albicans ATCC 22972	Xanthorrhizol	4.0	1.0	0.25	0.375	Synergistic
	Amphotericin B	1.0	0.125	0.125		
C. albicans (clinical)						
B2756	Xanthorrhizol	4.0	1.0	0.25	0.75	Indifference
	Amphotericin B	0.5	0.25	0.5		
B4381	Xanthorrhizol	4.0	0.5	0.125	0.375	Synergistic
	Amphotericin B	0.5	0.125	0.25		
C372	Xanthorrhizol	4.0	1.0	0.25	0.375	Synergistic
	Amphotericin B	0.5	0.25	0.125		
C372	Xanthorrhizol	2.0	0.25	0.125	0.375	Synergistic
	Amphotericin B	0.5	0.125	0.25		
P173	Xanthorrhizol	2.0	0.25	0.125	0.375	Synergistic
	Amphotericin B	0.5	0.125	0.25		
G61	Xanthorrhizol	4.0	0.5	0.125	0.25	Synergistic
	Amphotericin B	1.0	0.125	0.125		
C. glabrata ATCC 90525	Xanthorrhizol	8.0	1.0	0.125	0.25	Synergistic
	Amphotericin B	1.0	0.125	0.125		
C. glabrata (clinical)						
B869	Xanthorrhizol	1.0	0.25	0.25	0.5	Synergistic
	Amphotericin B	0.5	0.125	0.25		
B1226	Xanthorrhizol	4.0	0.25	0.125	0.375	Synergistic
	Amphotericin B	0.5	0.125	0.25		
B240	Xanthorrhizol	4.0	1.0	0.25	0.5	Synergistic
	Amphotericin B	1.0	0.25	0.25		
B532	Xanthorrhizol	2.0	0.25	0.125	0.375	Synergistic
	Amphotericin B	0.5	0.125	0.25		
B115	Xanthorrhizol	4.0	0.25	0.125	0.375	Synergistic
	Amphotericin B	0.5	0.125	0.25		
C. guilliermondii ATCC 20216	Xanthorrhizol	4.0	0.5	0.125	0.25	Synergistic
	Amphotericin B	1.0	0.125	0.125		
C. guilliermondii (clinical)						
C25	Xanthorrhizol	4.0	1.0	0.25	0.5	Synergistic
	Amphotericin B	0.5	0.125	0.25		
B5050	Xanthorrhizol	4.0	0.25	0.063	0.313	Synergistic
C. krusei	Amphotericin B	0.5	0.125	0.25		
ATCC 6258	Xanthorrhizol	4.0	0.5	0.125	0.25	Synergistic
C. parapsilosis	Amphotericin B	1.0	0.125	0.125		
ATCC 7330	Xanthorrhizol	16.0	2.0	0.125	0.25	Synergistic
	Amphotericin B	1.0	0.125	0.125		
C. parapsilosis (clinical)						
B1153	Xanthorrhizol	8.0	2.0	0.25	0.375	Synergistic
	Amphotericin B	1.0	0.125	0.125		
C276	Xanthorrhizol	16.0	2.0	0.125	0.25	Synergistic
	Amphotericin B	1.0	0.125	0.125		
B3421	Xanthorrhizol	8.0	2.0	0.125	0.375	Synergistic
	Amphotericin B	0.5	0.125	0.25		
B1089	Xanthorrhizol	8.0	4.0	0.5	1.0	Indifference
	Amphotericin B	0.5	0.125	0.5		
B392	Xanthorrhizol	8.0	2.0	0.125	0.625	Indifference
	Amphotericin B	0.5	0.25	0.5		
C. tropicalis	Xanthorrhizol	2.0	0.5	0.125	0.375	Synergistic
ATCC 42678	Amphotericin B	0.5	0.125	0.25		

FIC, fractional inhibitory concentration (FIC = MIC combination/MIC alone); FICI = FIC of xanthorrhizol + FIC of amphotericin B.

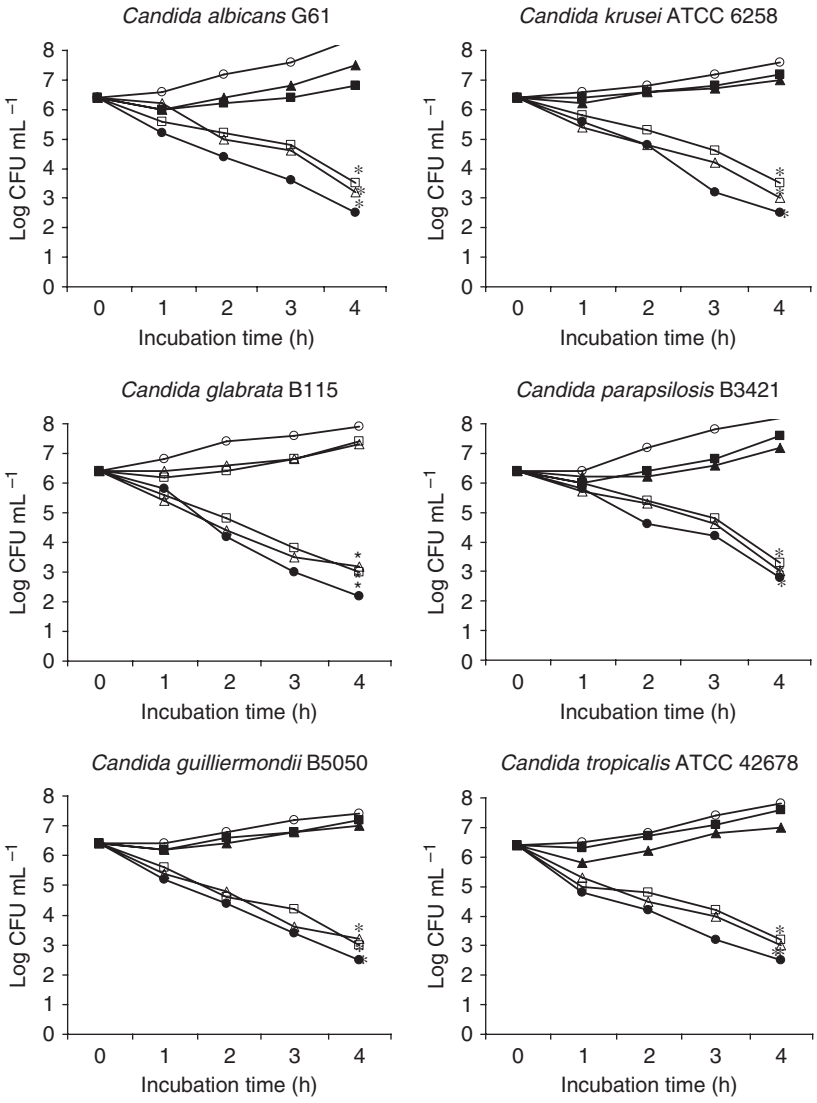

Fig. 3. Representative time–kill curves of 1/2 MIC dose of xanthorrhizol in combination with 1/2 MIC dose of ketoconazole. The *Candida* suspensions were cultured without anticandidal as a control (open circles), with 1/2 MIC dose of xanthorrhizol alone (filled squares), 1/2 MIC dose of ketoconazole alone (filled triangles), MIC of xanthorrhizol (open squares), MIC of ketoconazole (open triangles), and in combination (1/2 MIC dose of xamthorrhizol plus 1/2 MIC dose of ketoconazole) (filled circles). *Significant difference ($P < 0.05$) compared with the control.

with 1/2 MIC dose of ketoconazole against all *Candida* species tested and 1/2 MIC dose of xanthorrhizol in combination with 1/2 MIC dose of amphotericin B against *C. albicans*, *C. guilliermondii*, *C. krusei*, and *C. tropicalis* were fast acting; reduction in the number of CFU mL^{-1} was > 3 log units (99.9%). In general, these data demonstrate that xanthorrhizol has a synergistic anticandidal effect with ketoconazole or amphotericin B against *Candida* species.

Discussion

The potential antifungal effects of certain bioactive compounds from plants have attracted serious attention within the scientific community, largely as a result of the growing problem of multidrug resistance among pathogenic fungi and toxic effect of used therapeutics (Cowan, 1999; Shin & Pyun, 2004; Tim Cushnie & Lamb, 2005). In contrary, there are a few reports concerning the effect of natural antifungal agents isolated from plants in combination with commercial antifungal agents. Rukayadi *et al.* (2006) have reported that xanthorrhizol isolated from Javanese turmeric (*C. xanthorrhiza*) conferred significant antifungal activity. In this study, we report the anticandidal effect of xanthorrhizol in combination with the commonly used antifungal agents, ketoconazole, and amphotericin B.

With regard to the combined effects of antifungal agents *in vitro*, Tables 2 and 3 show arithmetic means of FICI values after 16 repetitions ($n = 4 \times 4$) per combination of compounds and per species. Mostly, the combined anticandidal effect of xanthorrhizol with ketoconazole or amphotericin B against all tested *Candida* was classified as synergistic

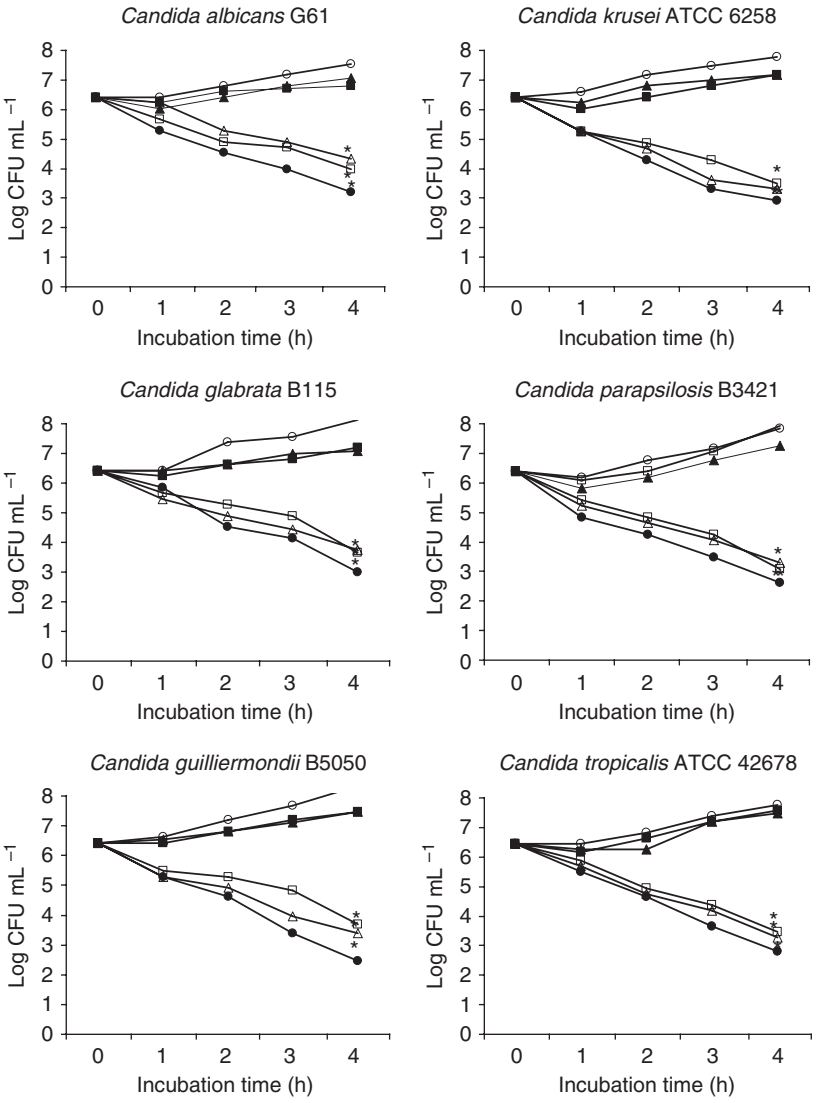

Fig. 4. Representative time–kill curves of 1/2 MIC dose of xanthorrhizol in combination with 1/2 MIC dose of amphotericin B. The *Candida* suspensions were cultured without anticandidal agent as a control (open circles), with 1/2 MIC dose of xanthorrhizole alone (filled squares), 1/2 MIC dose of amphotericin B alone (filled squares), MIC of xanthorrhizol (open squares), MIC of amphotericin B (open triangles), and in combination (1/2 MIC dose of xamthorrhizol plus 1/2 MIC dose of amphotericin B) (filled circles). *Significant difference ($P < 0.05$) compared with the control.

regardless of the values used for FICI calculation (Shin & Lim, 2004; Karpanen *et al*., 2008). No differences were found for the combination of xanthorrhizol with amphotericin B against a clinical *C. albicans* B2756 isolate and clinical isolates of *C. parapsilosis* (B1089 and B392). No differences were also found for xanthorrhizol with ketoconazole combination against a clinical *C. albicans* P173 isolate, a clinical *C. glabrata* B240 isolate and clinical isolates of *C. parapsilosis* (B1153 and B1089). The increase in available antifungal compounds isolated from natural products has prompted search for better efficacy strategies, such as using the antifungal agents isolated from edible medicinal plants in combination with common antifungals (Cuenca-Estrella, 2004). Furthermore the synergistic combinations of antifungal agents may have positive effects. For instance, antifungal combination therapies may increase the rate of fungal killing, shorten the duration of therapy, avoid the emergence of drug resistance, expand the spectrum of activity, and decrease drug-related toxicity by allowing the use of lower doses of antifungal agents (Polak, 2000; Zhu *et al*., 2004). Xanthorrhizol in combination with ketoconazole or amphotericin B showed $\geq$83% synergistic effect against six species of *Candida* with a total number of 24 strains (Tables 2 and 3). Thus, xanthorrhizol in combination with ketoconazole or amphotericin might have positive effects for the treatment of candidal infections. Other beneficial effects of synergistic combinations of antifungal agents would accrue, *Candida* infections with one drug clearing one body system while the other clears it from a different body site (Cuenca-Estrella, 2004). Ketoconazole inhibits ergosterol biosynthesis, a bioregulator of membrane fluidity and asymmetry and consequently of membrane integrity in fungal cells, by

the inhibition of 14-α-demethylase. Amphotericin B binds directly to ergosterol to alter cell membrane activity in fungal cells (Ghannoum & Rice, 1999). In contrast, xanthorrhizol affect the external morphology of the yeast cells, such as clumpiness, abnormalities, protrusions, and become wrinkled on the cell surface of *C. albicans*, *C. glabrata*, *C. guilliermondii*, and *C. parapsilosis*, respectively (Rukayadi & Hwang, 2007a). However, the action of xanthorrhizol on *Candida* cell is still not clear. Future research toward action mechanism of xanthorrhizol on *Candida* cell may resolve this issue.

Han (2007) reported that reduction of the concentrations of common antifungal agents, such as ketoconazole or amphotericin B, by combining with anticandidal activity may be very important. In our study, xanthorrhizol could reduce the concentrations of both ketoconazole and amphotericin B. The growth of all *Candida* species tested can be inhibited by ketoconazole at MICs of 0.5–1.0, 0.5–1.0, 0.5–1.0, 1.0, 0.5–1.0, and 1.0 $\mu g\,mL^{-1}$ for *C. albicans*, *C. glabrata*, *C. guilliermondii*, *C. parapsilosis*, *C. krusei*, and *C. tropicalis*, respectively (Table 1). In combination with xanthorrhizol, the concentration of ketoconazole for inhibiting the growth of the tested *Candida* species could be reduced $\geq$50%; concentrations of ketoconazole were 0.125–0.25, 0.125–0.5, 0.125, 0.125, 0.125, and 0.125 $\mu g\,mL^{-1}$, respectively (Table 2). Amphotericin B at MICs of 0.5–1.0, 0.5–1.0, 0.5–1.0, 1.0, 0.5–1, and 0.5 $\mu g\,mL^{-1}$ inhibited growth of *C. albicans*, *C. glabrata*, *C. guilliermondii*, *C. parapsilosis*, *C. krusei*, and *C. tropicalis*, respectively (Table 1). In combination with xanthorrhizol, the concentration of amphotericin B that inhibited the growth of the tested *Candida* species was 0.125–0.25, 0.125–0.25, 0.125, 0.125, 0.125–0.25, and 0.125 $\mu g\,mL^{-1}$, respectively (Table 3). These results indicated that the use of xanthorrhizol allows the reduction of the concentration of ketoconazole and amphotericin B to inhibit the growth of all *Candida* species tested. Antifungal compounds used in combination might promote the effectiveness of each drug, with efficacy being achieved using a lower dose of each drug (Cuenca-Estrella, 2004). Moreover, the combination of commercial antifungals with another antifungal activity may be fast acting against fungal pathogens if compared with single antifungals (Nooney *et al.*, 2005). Our study presented that fungicidal activity of 1/2 MIC dose of xanthorrhizol in combination with 1/2 MIC dose of ketoconazole against all *Candida* tested and 1/2 MIC dose of xanthorrhizol in combination with 1/2 MIC dose of amphotericin B against *C. albicans*, *C. guilliermondii*, *C. krusei*, and *C. tropicalis* were fast acting; reduction in the number of CFU mL^{-1} was $>$ 3 log units (99.9%) at endpoint $\leq$ 4 h (Figs 3 and 4).

For treatment of fungal infections, amphotericin B and azoles drugs have been considered as the drug of choice and are mainly used in common clinical practice (Gallis *et al.*, 1990; Hartsel & Bolard, 1996). However, toxicity and resistance to these antifungal drugs are major problems (Edwards, 1991). Furthermore, a long-term treatment with the commonly used antifungal agent, such as amphotericin B or ketoconazole alone or combined, has toxic effect (Denning *et al.*, 1997; Fonos & Cataldi, 2000; Mayer *et al.*, 2002). Xanthorrhizol, as a natural and active compound isolated from an edible medicinal plant, showing most synergistic anticandidal activity effect in combination with ketoconazole or amphotericin, which might be effective for the treatment of candidal infections. These *in vitro* results should be confirmed by *in vivo* studies or by clinical evidence to validate the practicality of the therapeutic application of these combinations.

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