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In vitro anticandidal activity of xanthorrhizol isolated from *Curcuma xanthorrhiza* Roxb

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***Objectives*: Xanthorrhizol, isolated from the methanol extract of *Curcuma xanthorrhiza* Roxb., was investigated for its anticandidal activity using six *Candida* species.**

***Methods*: The *in vitro* susceptibility tests for xanthorrhizol were carried out in terms of MIC and minimal fungicidal concentration (MFC) using the NCCLS M27-A2 broth microdilution method. Time–kill curves were determined to assess the correlation between MIC and fungicidal activity of xanthorrhizol at concentrations ranging from 0 MIC to 4× MIC.**

***Results*: All *Candida* species showed susceptibility to xanthorrhizol in the MIC range 1.0–15.0 mg/L for *Candida albicans*, 1.0–10 mg/L for *Candida glabrata*, 2.0–8.0 mg/L for *Candida guilliermondii*, 2.5–7.5 mg/L for *Candida krusei*, 2.5–25 mg/L for *Candida parapsilosis* and 2.0–8.0 mg/L for *Candida tropicalis*. Time–kill curves demonstrated that xanthorrhizol was able to kill the *Candida* strains with MFCs of 20 mg/mL, 15 mg/mL, 12.5 mg/mL, 10 mg/L, 30 mg/mL and 10 mg/L for *C. albicans*, *C. glabrata*, *C. guilliermondii*, *C. krusei*, *C. parapsilosis* and *C. tropicalis*, respectively.**

***Conclusions*: The potent anticandidal activity of xanthorrhizol may support the use of *C. xanthorriza* for the treatment of candidiasis.**

Keywords: *C. xanthorriza*, MIC, MFC, antifungals

Introduction

Candida species are now recognized as a significant cause of hospital-acquired infection. *Candida albicans* is the organism most often associated with serious fungal infection and it is showing increased resistance to traditional antifungal agents.[1] Recently, non-*C. albicans* species, such as *Candida glabrata*, *Candida guilliermondii*, *Candida krusei*, *Candida parapsilosis* and *Candida tropicalis*, have also shown dramatic increases in fungal infections and antifungal resistance.[2]

The development of resistance in known fungal pathogens and emergence of new fungal pathogens intrinsically resistant to the currently available antibiotics demonstrate the urgent importance of identifying novel antifungal agents. The antifungal effect of essential oils of many plants and their specific anticandidal activity are well established.[3] *Curcuma xanthorrhiza* Roxb., commonly known as Javanese turmeric, has been traditionally used in South-East Asian countries for food and medicinal purposes. Xanthorrhizol, isolated from *C. xanthorrhiza*, has been reported to possess anticariogenic activity against *Streptococcus mutans*.[4] In the present research, we found that xanthorrhizol exhibited antifungal activity against *C. albicans*, *C. glabrata*, *C. guilliermondii*, *C. krusei*, *C. parapsilosis* and *C. tropicalis*. To our knowledge, this is the first report showing that xanthorrhizol possesses anticandidal activity against pathogenic non-*C. albicans* species.

Materials and methods

Candida isolates, growth conditions and inoculum

The reference *Candida* species (*C. albicans*, *C. glabrata*, *C. guilliermondii*, *C. krusei*, *C. parapsilosis* and *C. tropicalis*) used in this study were all obtained from the American Type Culture Collection (Rockville, MD, USA). Clinical *Candida* isolates were obtained from

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The Research Institute of Bacterial Resistance, College of Medicine, Yonsei University, Korea. The clinical isolates were from body fluid, blood, urine, sputum and genitals of the patients. The species were identified by conventional methods or using Vitex YBC cards (bioMérieux, Marcy l'Étoile, France), according to the manufacturer's instructions. All clinical *Candida* isolates were susceptible to fluconazole and voriconazole. The strains were cultured in Sabouraud dextrose broth (SDB) or on Sabouraud dextrose agar (SDA) (Difco) for 48 h at 35°C. A standardized inoculum (a McFarland standard) for each isolate was 5×10^6 cfu/mL.[5]

Anticandidal agents

Xanthorrhizol was isolated as the pure form from the ethylacetate fraction of the methanol extract of *C. xanthorrhiza* according to the method of Hwang *et al*.[4] Xanthorrhizol was dissolved in 10% dimethylsulfoxide (DMSO) to obtain 1 g/L stock solutions. DMSO at 10% was found not to kill the *Candida*. Amphotericin B, purchased from Sigma Chemical Co. (St Louis, MO, USA), was dissolved in sterile distilled water to obtain 1 g/L stock solution. The concentrations of individual antifungal agents ranged as follows: xanthorrhizol, 10–1000 mg/L; amphotericin B, 1–75 mg/L.

In vitro susceptibility tests

In vitro susceptibility tests were performed to evaluate MICs and minimal fungicidal concentrations (MFCs) using the method described in the guidelines of NCCLS M27-A2.[5] Briefly, MICs were determined for all species with the adjusted inoculum suspension of 5×10^6 cfu/mL by diluting the suspension 1:100 with MOPS-buffered RPMI 1640 medium to give a final inoculum concentration of 5×10^4 cfu/mL. Individual antifungal agents were diluted 1:10 in MOPS-buffered RPMI 1640 medium containing 5×10^4 cfu/mL inoculum, yielding an initial inoculum of 4.5×10^3 cfu/mL. The final concentration of individual antifungal agents ranged as follows: xanthorrhizol, 1–100 mg/L; amphotericin B, 0.1–7.5 mg/L. A 200 µL aliquot of each suspension was placed in 96-well round-bottomed microtitration plates. The plates were incubated at 35°C, and endpoints were read visually after 48 h. MFCs were determined for each anticandidal agent/*Candida* species/medium combination as outlined for MICs by removing the medium from each well showing no visible growth and subculturing onto SDA plates. The plates were incubated at 35°C until growth was seen in the growth control plates.

Time–kill curves

Time–kill assays were performed in standard MOPS-buffered RPMI 1640.[5] Before the tests were performed, the *Candida* species were subcultured at least twice and grown for 24 h at 35°C on SDA plates. The adjusted inoculum suspension of 5×10^6 cfu/mL was diluted 1:10 in MOPS-buffered RPMI 1640 medium to give a final inoculum concentration of 5×10^5 cfu/mL. Each concentration of xanthorrhizol was diluted 1:10 in MOPS-buffered RPMI 1640 medium containing 5×10^5 cfu/mL. This procedure yielded an initial inoculum of 4.5×10^5 cfu/mL. Final concentrations of xanthorrhizol were 0× MIC, 0.5× MIC, MIC, 2× MIC and 4× MIC for each *Candida* species. Cultures (5 mL final volume) were incubated at 35°C with agitation (200 rpm). At pre-determined time points (0, 2, 4, 8, 12, 24 and 48 h) 100 µL aliquots were 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 xanthorrhizol-free cells. Pellets were suspended in 100 µL of sterile distilled water and serially diluted. An appropriate volume (100, 40 or 20 µL, depending on the dilution and the concentration of xanthorrhizol) was spread onto SDA plates and incubated at 35°C for 48 h or more (until the colonies were seen on the plates) to determine the numbers of cfu/mL.

Results

MICs and MFCs of xanthorrhizol compared with those of amphotericin B are summarized in Table 1. All species were susceptible to xanthorrhizol. Interestingly, there were three strains of clinical *C. albicans* and one strain each of clinical *C. glabrata* and clinical *C. parapsilosis* that were not killed by more than 20 mg/L amphotericin B, but the strains were killed by xanthorrhizol, at 5.0–10.0 mg/L for clinical *C. albicans*, 5.0 mg/L for clinical *C. glabrata* and 7.5 mg/L for clinical *C. parapsilosis*.

The killing activity of xanthorrhizol for each species is represented in Figure 1. The fungicidal activity of xanthorrhizol was fast acting against all *Candida* tested; the reduction in the number of cfu/mL was >3 log units (99.9%). The fungicidal endpoints for *C. albican*s and *C. parapsilosis* were reached after 48 h of incubation at 4× MIC, while those for *C. guilliermondii*, *C. krusei* and *C. tropicalis* were reached after 12 h, and that for *C. glabrata* was reached after 24 h, at 4× MIC. The strongest fungicidal activity of xanthorrhizol was observed for *C. guilliermondi*, *C. krusei* and *C. tropicalis*. The fungicidal endpoint of *C. guilliermondii*, *C. krusei and C. tropicalis* was reached after 12 h at 4× MIC and after 24 h at 2× MIC. These data demonstrated that the ability to kill *Candida* species is dependent on the concentration of xanthorrhizol and the species.

Discussion

In this research, we used MOPS-buffered RPMI 1640 medium for measuring the MICs of amphotericin B and xanthorrhizol. The results showed that MICs of amphotericin are high, particularly for *C. parapsilosis*. A broth microdilution technique using RPMI 1640 is suitable for most antifungals except for amphotericin B, as apparent resistance to this drug is not always detected in RPMI 1640. MOPS-buffered RPMI 1640 is not an ideal medium for measuring the MICs of amphotericin.[5]

Generally, MICs and MFCs of amphotericin B against *Candida* species are in good agreement with previous reports.[2,6] The isolates with reduced susceptibility to amphotericin B may also exhibit reduced susceptibility to xanthorrhizol. There was no previous report for xanthorrhizol against pathogenic non-*C. albicans* species. In the present study xanthorrhizol has been shown to possess powerful *in vitro* activity against a broad range of distantly related *Candida* species. An anticandidal which has broad-spectrum activity against non-*C. albicans* species may have clinical applications. Snydman[7] reported that the spectrum of invasive fungal infections is changing and rising with the frequencies of infections due to non-*C. albicans* species.

Among the six species of *Candida* used in this study, *C. guilliermondii*, *C. krusei* and *C. tropicalis* were the most susceptible to xanthorrhizol followed by *C. glabrata*, *C. albicans* and *C. parapsilosis.* Knowledge of the *in vitro* pharmacodynamic characteristics of *C. guilliermondii* is still poor and limited to amphotericin B.[8] *C. glabrata* infection is second or third in frequency after *C. albicans* and is associated with a high mortality rate in at-risk hospitalized patients. However, its epidemiology, pathogenesis, treatment and antifungal resistance are poorly understood.[7] Moreover, compared with other *Candida* species, especially with *C. albicans*, higher levels of all azole antifungals

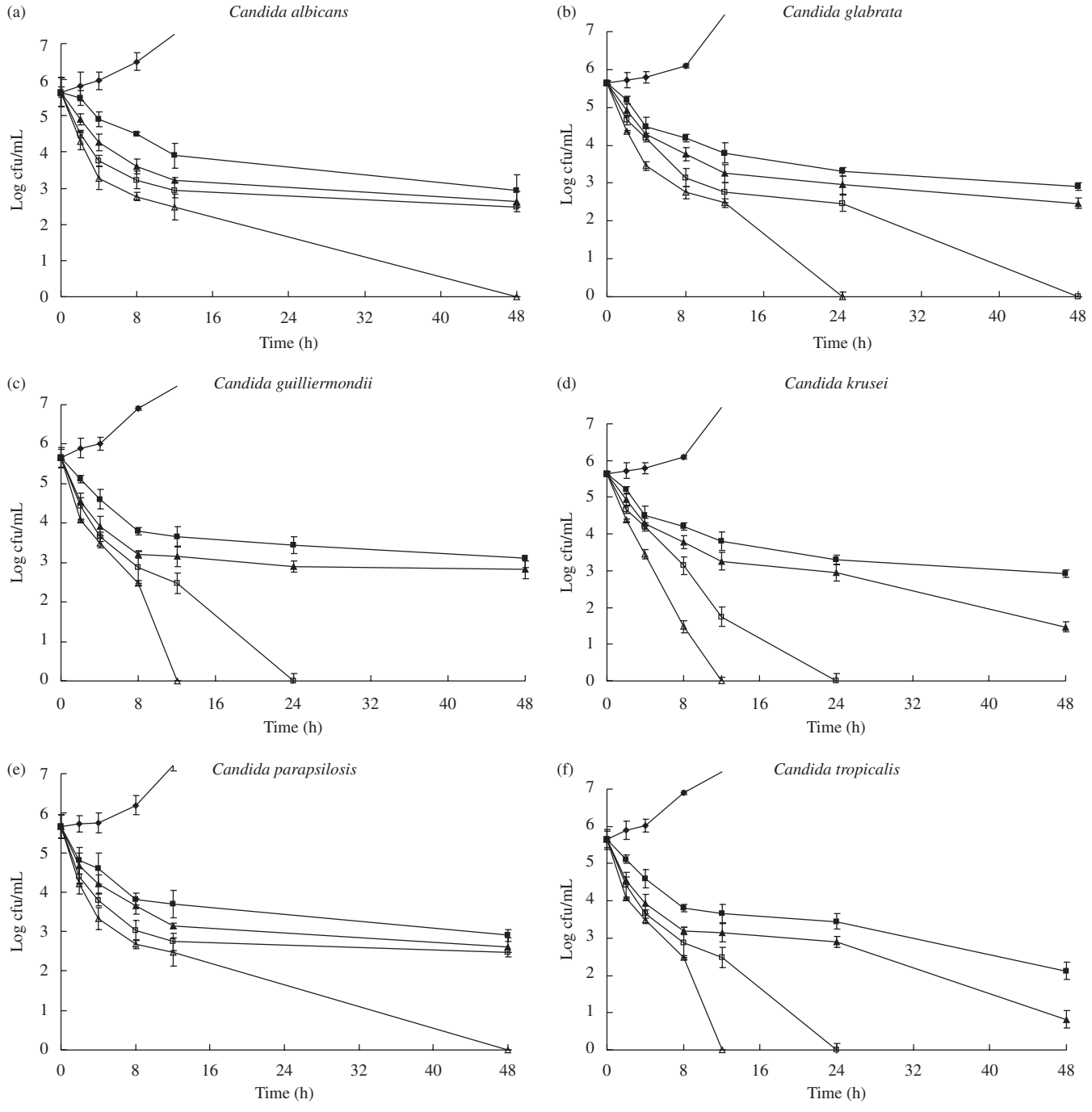

Figure 1. Representative time–kill plots for *Candida* species following exposure to xanthorrhizol at 0× MIC (filled diamonds), 0.5× MIC (filled squares), 1× MIC (filled triangles), 2× MIC (open squares) and 4× MIC (open triangles) after endpoint (48 h). (a) *C. albicans* ATCC 10231 (0, 7.5, 15, 30 and 60 mg/L); (b) *C. glabrata* ATCC 50044 (0, 5, 10, 20 and 40 mg/L); (c) *C. guilliermondii* ATCC 9058 (0, 4, 8, 16 and 32 mg/L); (d) *C. krusei* ATCC 32196 (0, 3.75, 7.5, 15 and 30 mg/L); (e) *C. parapsilosis* ATCC 22019 (0, 12.5, 25, 50 and 100 mg/L); and (f) *C. tropicalis* ATCC 28142 (0, 4, 8, 16 and 32 mg/L). Values given in the brackets after species are 0× MIC, 1× MIC, 2× MIC and 4× MIC, respectively.

are required for comparable activity against *C. glabrata* isolates.[9] Our study showed that *C. glabrata* was more susceptible to xanthorrhizol compared with *C. albicans*. This result suggested that xanthorrhizol may be effective in treating *C. glabrata* infection. *C. parapsilosis* is the most frequently isolated yeast species from hospital patients and the most common non-*C. albicans* species causing infection in cancer patients.[10] Our results also demonstrated that xanthorrhizol has the ability to inhibit and to kill *C. parapsilosis in vitro*.

Overall, xanthorrhizol may be potentially valuable as a natural compound for fungal infections, even though its anticandidal activity is weaker than that of amphotericin B against six species of *Candida*. However, the clinical applications of xanthorrhizol are challenging to interpret in this study due to a lack of

Table 1. *In vitro* anticandidal activity of xanthorrhizol against *Candida* species

Drug/*Candida* spp.	No. of isolates tested for MIC/MFC	MIC (mg/L)			MFC (mg/L)		
		MIC_{50}	MIC_{90}	range	MFC_{50}	MFC_{90}	range
Xanthorrhizol							
C. albicans ATCC	12	5.0	10.0	2.5–15	8.0	8.0	5.0–20.0
C. albicans (clinical)	18	2.5	4.0	1.0–5.0	6.0	6.0	5.0–10.0
C. albicans (clinical)[a]	3	2.5	4.0	2.0–5.0	5.0	5.0	5.0–10.0
C. glabrata (ATCC)	4	5.0	8.0	4.0–10	10.0	10.0	7.5–15.0
C. glabrata (clinical)	11	2.5	4.0	1.0–5.0	4.0	4.0	2.5–7.5
C. glabrata (clinical)[a]	1			2.5			5.0
C. guilliermondii (ATCC)	4	4.0	6.0	2.0–8.0	7.5	7.5	5.0–12.5
C. guilliermondii (clinical)	2			2.5 and 4.0			5.0 and 7.5
C. krusei (ATCC)	4	4.0	7.5	2.5–7.5	5.0	5.0	4.0 and 10.0
C. parapsilosis (ATCC)	4	12.5	17.5	10.0–25	20.0	20.0	15–30
C. parapsilosis (clinical)	19	10	15	7.5–20	20.0	20.0	15–30
C. parapsilosis (clinical)[a]	1			5.0			7.5
C. tropicalis (ATCC)	4	4.0	6.0	2.0–8.0	5.0	5.0	4.0–10.0
Amphotericin B							
C. albicans ATCC	12	1.5	2.5	1.0–4.0	2.5	5.0	1.5–7.5
C. albicans (clinical)	18	1.0	2.0	0.5–2.5	2.5	4.0	1.0–5.0
C. albicans (clinical)[a]	3			> 15			> 20
C. glabrata (ATCC)	4	2.0	4.0	1.5–5.0	4.0	6.0	2.0–8.0
C. glabrata (clinical)	11	1.0	2.0	0.5–2.5	2.5	4.0	1.0–5.0
C. glabrata (clinical)[a]	1			> 15			> 20
C. guilliermondii (ATCC)	4	4.0	7.5	2.5–7.5	8.0	10.0	7.5–12.5
C. guilliermondii (clinical)	2			2.5 and 5.0			2.5 and 5.0
C. krusei (ATCC)	4	2.0	4.0	1.0–5.0	4.0	7.5	2.0 and 7.5
C. parapsilosis (ATCC)	4	5.0	7.5	4.0–125	7.5	10.0	5.0–15
C. parapsilosis (clinical)	19	5.0	7.5	7.5–20	7.5	10.0	5.0–15
C. parapsilosis (clinical)[a]	1			>15			>20
C. tropicalis (ATCC)	4	2.5	4.0	2.0–8.0	4.0	5.0	2.5–7.5

[a]Isolates resistant to amphotericin B.

pharmacokinetic and safety studies. Unanswered concerns exist about the *in vivo* efficacy and toxicity of xanthorrhixol. Future research towards these objectives, based on animal models, may resolve these issues.

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Transparency declarations

None to declare.

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