Inhibitory effects of sugiol, a biologically active abietane type diterpenoid from *Metasequoia glyptostroboides*

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VIVEK K. BAJPAI¹, KWANG-HYUN BAEK¹, SUN CHUL KANG²,*
¹ School of Biotechnology, Yeungnam University, Gyeongsan, Gyeongbuk 712-749, Republic of Korea
² Department of Biotechnology, Daegu University, Gyeongsan, Gyeongbuk, 712-714, Republic of Korea

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*To whom correspondence should be addressed:
Prof. Sun Chul Kang; E-mail: sckang@daegu.ac.kr; Fax: +82-53-850-6559

Abstract

This study was aimed to evaluate the antifungal effects of an abietane type diterpenoid, sugiol isolated from *Metasequoia glyptostroboides* against different clinical isolates of Candida species. The sugiol (100 μg/disc) evoked potential antifungal effect as a diameter of zones of inhibition against Candida albicans KBN06P00076, C. albicans KBN06P00074, C. glabrata KBN06P00066, C. glabrata KBN06P00068, C. tropicalis KBN06P00058, C. parapsilosis KBN06P00060, C. parapsilosis KBN06P00055 and C. guilliermondii KBN06P00492, C. guilliermondii KBN06P00867, which was ranged from 8 to 13 mm. The minimum inhibitory (MIC) and minimum fungicidal (MFC) concentrations values of sugiol against the employed fungal pathogens were found in the range of 125 to 1000 and 125 to 2000 μg/ml, respectively. Also the sugiol exerted a remarkable antifungal effect on the viable counts of the tested fungal pathogens. These results demonstrate potent therapeutic efficacy of a diterpenoid compound sugiol from *M. glyptostroboides*.

Keywords: *Metasequoia glyptostroboides*; Sugiol; Antifungal effect; *Candida* species; Clinical isolates;

Introduction

Systemic fungal infections have emerged as important causes of morbidity and mortality in immunocompromised patients including AIDS, cancer chemotherapy, and organ or bone marrow transplantation. *Candida* species are ubiquitous fungi that represent the most common fungal pathogens that affect humans. The growing problem of mucosal and systemic candidiasis reflects the enormous increase in the number of patients at risk and the increased opportunity that exists for *Candida* species to invade deep tissues.

The increased prevalence of local and systemic disease caused by *Candida* species has resulted in numerous new clinical syndromes. *Candida* species produce a wide spectrum of diseases, ranging from superficial mucocutaneous disease to invasive illnesses, such as hepatosplenic candidiasis, *Candida* peritonitis, and systemic candidiasis [1]. The management of serious and life-threatening invasive candidiasis remains severely hampered by the lack of reliable antifungal drugs that allow both fungemia and tissue invasion by *Candida* species. Patients who are critically ill or immunosuppressed and in medical and surgical ICUs have been the prime targets for opportunistic nosocomial fungal infections, primarily due to *Candida* species. In persons with systemic infections, *Candida* species are now the fourth most commonly isolated pathogens from blood cultures [2]. Clinical and autopsy studies have
confirmed the marked increase in the incidence of disseminated candidiasis, reflecting a parallel increase in the frequency of candidemia [3,4].

Despite advances in antifungal therapy, the treatment of infections caused by *Candida* species with amphotericin B, the azoles or flucytosine has not been uniformly successful. In the 1990s, many HIV infected patients received long-term, low-level azole antifungal therapy, which resulted in azole-resistant isolates of *Candida* species [5]. The management of *Candida* infections faces a number of problems including limited number of effective antifungal drugs, toxicity of the available antifungal drugs, increased resistance of *Candida* to commonly used antifungal drugs, relapse of *Candida* infections and the high cost of antifungal drugs [6-8].

In order to overcome these problems, plant-based bioactive compounds have been exploited to serve as potent bio-medicinal tools for using in medicine industry to control serious fungal infection caused by *Candida* species. Studies suggested that plant-based bioactive terpenoids have been found to possess substantial antifungal effect against various fungal isolates of *Candida* species [9-11].

In this study described herein, sugiol (Figure 1), an abietane type diterpenoid previously isolated from *Metasequoia glyptostroboides*, was evaluated in order to confirm its therapeutic antifungal efficacy against several clinical isolates of *Candida* species.

**Figure 1.** Chemical structure of sugiol, a biologically active diterpenoid from *M. glyptostroboides*.

**Materials and Methods**

**Fungal pathogens**

The test fungal isolates of *Candida* species such as *Candida albicans* KBN06P00076, *C. albicans* KBN06P00074, *C. glabrata* KBN06P00066, *C. glabrata* KBN06P00068, *C. tropicalis* KBN06P00682, *C. tropicalis* KBN06P00058, *C. parapsilosis* KBN06P00060, *C. parapsilosis* KBN06P00055, *C. guilliermondii* KBN06P00492 and *C. guilliermondii* KBN06P00867 were provided by the National Biobank of Korea, Chonbuk National University Hospital, supported by the Ministry of Health, Welfare and Family Affairs, Republic of Korea. All materials derived from the National Biobank of Korea were obtained with informed consent under institutional review board-approved protocols.

**Antifungal activity assay**

A standard agar diffusion method was employed to determine antifungal activity [12]. Briefly, a 100 μl of standardized inoculum containing 10^7 CFU/ml of fungal suspension was loaded uniformly on petri plates with 20 ml of potato-dextrose agar (PDA) medium, and allowed to dry for 5 min. A sterile Whatman No. 1 filter paper disc with 6 mm diameter was impregnated with 100 μg/disc of test compound sugiol dissolved in 5% dimethyl sulphoxide (DMSO). Negative controls were prepared using the same solvent employed to dissolve the sample. Standard reference antibiotic, amphotericin B (10 μg/disc, Sigma-Aldrich Co., St.

Louis, MO, USA) was used as the positive control against the tested clinical isolates of *Candida* species. After incubating the plates at 28°C for 2 - 3 days, antifungal activity was evaluated by comparing the diameter of the zones of inhibition against the tested *Candida* species. For the antifungal activity assay, the experiment was replicated at least three times.

**Minimum inhibitory (MIC) and minimum fungicidal (MFC) concentrations**

A two-fold serial dilution method was used to test the MIC of sugiol [13]. The sugiol dissolved in 5% DMSO was incorporated into PDB media to obtain a concentration of 2000 μg/ml, and then serially diluted to achieve 1000, 500, 250, 125, 62.5, 31.25, 15.62 and 7.81 μg/ml. A 10 μl standardized suspension of each tested *Candida* species (10^7 CFU/ml approximately) was transferred to each tube. The control tubes with only candidal suspension were incubated at 28°C for 2 - 3 days. MIC expressed in μg/ml was defined as the lowest concentration of the sugiol not showing any growth of test *Candida* species by macroscopic evaluation. In addition, the concentrations showing complete inhibition of visual growth of fungal isolates were identified, and 50 μl of each diluted culture broth transferred on to the agar plates were incubated for specified time and at temperature as mentioned above. The complete absence of growth on the agar surface in the lowest concentration of sample was defined as MFC.

**Cell viability assay**

For cell viability assay, active cultures were prepared in PDB medium [14]. Each of the tubes containing re-suspended candidal suspension (approximately 10^7 CFU/ml) was inoculated with 125 μg/ml concentration of the test compound sugiol in 10 ml PDB broth, and incubated at 28°C. For viable cell counts, samples were taken out at 0, 20, 40, 60, 80, 100 and 140 min time intervals, and the viable cells were counted as followed: After incubation, 1 ml of re-suspended culture was diluted into 9 ml buffer peptone water, there by diluting it 10-fold. A 0.1 ml sample of each treatment was diluted further and spread on the surface of PDA agar medium. Newly formed colonies were counted after 2-3 days of incubation at 28°C. The controls were composed of inoculums without test compound for each *Candida* isolate with same experimental conditions. Each assay in this experiment was replicated three times.

**Statistical analysis**

Each experiment was run in triplicate and the average values were calculated. The statistical analysis was carried out employing one way ANOVA (p < 0.05) with a SPSS statistical package (version 11.0).

**Results**

**Antifungal effect of sugiol**

The antifungal effect of sugiol against the employed isolates of *Candida* species was monitored by the presence of diameter of inhibition zones. The sugiol (100 μg/disc) exhibited potent inhibitory effect (Figure 2). *C. albicans* KBN06P00076, KBN06P00074, *C. tropicalis* KBN06P00682, KBN06P00058, *C. parapsilosis* KBN06P00060 and KBN06P00055 were found to be the most inhibited fungal pathogens by the sugiol, with diameter of zones of inhibition of 13, 12, 13, 13 and 12 mm, respectively. However, the diameters of zones of inhibition of sugiol against *C. glabrata* KBN06P00066, KBN06P00068, *C. guilliermondii* KBN06P00492 and KBN06P00867 were found to be 11, 9, 9 and 8 mm, respectively (Figure 2). Only solvent as a negative control did not show any antifungal effect. It was confirmed in this assay that sugiol significantly inhibited the growth of some of the tested clinical isolates of *Candida* species (diameter of zones of inhibition: 8 to 13 mm) than that of standard antibiotic amphotericin B (diameter of zones of inhibition: 12 to 14 mm) (Figure 2).
Figure 2. Antifungal effect of sugiol (100 \( \mu \text{g/disc} \)) against clinical isolates of \textit{Candida} species. Ca-1: \textit{Candida albicans} KBN06P00076, Ca-2: \textit{C. albicans} KBN06P00074, Cg-1: \textit{Candida glabrata} KBN06P00066, Cg-2: \textit{C. glabrata} KBN06P00068, Ct-1: \textit{Candida tropicalis} KBN06P00682, Ct-2: \textit{C. tropicalis} KBN06P00058, Cp-1: \textit{Candida parapsilosis} KBN06P00060, Cp-2: \textit{C. parapsilosis} KBN06P00055, Cgl-1: \textit{Candida guilliermondii} KBN06P00492 and Cgl-2: \textit{C. guilliermondii} KBN06P00867.

\textbf{MIC and MFC}

The sugiol showed potent inhibitory effect as MIC and MFC values against the tested clinical isolates of \textit{Candida} species. The MIC and MFC values of sugiol against the tested isolates were found in the range of 125 to 1000 and 125 to 2000 \( \mu \text{g/ml} \), respectively (Figure 3). On the other hand, the MIC values of standard amphotericin B against the tested isolates of \textit{Candida} species were noted in the range of 62.5 to 250 \( \mu \text{g/ml} \). Clinical isolates of \textit{C. guilliermondii} KBN06P00492 and KBN06P00867 were found less sensitive to amphotericin B as compared to the other isolates. In addition, \textit{C. albicans} KBN06P00076, KBN06P00074, \textit{C. tropicalis} KBN06P00682, KBN06P00058 and \textit{C. parapsilosis} KBN06P00060 were found to be the most susceptible \textit{Candida} species to the sugiol (MIC: 125 ~ 250 \( \mu \text{g/ml} \)) followed by \textit{C. glabrata} KBN06P00066, KBN06P00068, \textit{C. parapsilosis} KBN06P00055, \textit{C. guilliermondii} KBN06P00492 and KBN06P00867 (MIC: 500 ~ 1000 \( \mu \text{g/ml} \)).

Figure 3. Minimum inhibitory (MIC) and minimum fungicidal (MFC) concentrations of sugiol against clinical isolates of \textit{Candida} species.
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Ca-1: *Candida albicans* KBN06P00076, Ca-2: *C. albicans* KBN06P00074, Cg-1: *Candida glabrata* KBN06P00066, Cg-2: *C. glabrata* KBN06P00068, Ct-1: *Candida tropicalis* KBN06P000682, Ct-2: *C. tropicalis* KBN06P00058, Cp-1: *Candida parapsilosis* KBN06P00060, Cp-2: *C. parapsilosis* KBN06P00055, Cgl-1: *Candida guilliermondii* KBN06P00492 and Cgl-2: *C. guilliermondii* KBN06P00867.

**Effect of sugiol on viable cell counts**

To evaluate the antifungal effect of sugiol on some of the selected clinical isolates of *Candida* species, a viable cell count assay was performed. The sugiol had potent inhibitory effect on the growth of tested fungal isolates at the used concentrations. At 100 min exposure, near about 80-95% inhibition of the viable cells was observed in all the tested isolates (Figure 4). However, sugiol completely inhibited the cell viable count numbers against *C. albicans* KBN06P00076, *C. glabrata* KBN06P00066, KBN06P00058 and *C. parapsilosis* KBN06P00060 at 140 min exposure time, and no colony forming units were observed.
Discussion

In this research we demonstrated the antifungal efficacy of an abietane type diterpenoid compound sugiol, inhibiting the growth of yeast-like fungal pathogens of Candida species. The data of this study suggest that sugiol may act as an effective agent when applied to a clinical Candida infection situation. Only solvent as a negative control had no antifungal effect against any of the tested fungal isolates. The sugiol also had a complex effect on the antifungal activity as MIC and MFC values as well as its inhibitory effect on cell viable counts of the tested clinical isolates of Candida species. The sugiol, an abietane type diterpenoid, exhibited a wide range of antifungal activity against Candida species. Various plant-based terpenoid compounds have been shown to exert potent antifungal effect against the pathogenic fungal isolates of Candida species [9-11]. Previously it has been reported that diterpenoids derived from Trigonostemon chinensis and Phlomis displayed potential antifungal effect against some Candida species [15,16]. These findings suggest that various plant-based bioactive terpenoid compounds can be available for trials to control severe fungal infections caused by various pathogenic isolates of Candida species. Certain terpenoid compounds act in many ways on various types of disease complex, or may be applied in the same way as alternative medicinal products in a
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wide range of activities against the pathogenic microbes where these pathogens have developed resistance against the specific fungicides [15,17]. Information on the antifungal effects of terpenoid compounds is scant, and these results show, for the first time that sugiol possessed substantial antifungal effect against *Candida* species. *Candida* species are getting serious worldwide due to pathogenic disorders in human beings, although control measures are available but limitedly effective [18]. Hence, plant-based terpenoid compounds may be considered as effective alternatives to develop new and novel types of antifungal agents for preventive treatment of serious fungal infections in human beings caused by *Candida* species.

Conclusion

Antifungal effect of terpenoid compounds against pathogenic isolates of *Candida* species may offer many new applications of clinical trials. Plant-based bioactive terpenoids can be very sensitive tools in medicine industry and are highly desired with significant antifungal potential. The availability of such types of bioactive molecules may contribute to providing sustainable antifungal tools to inhibit the growth of medically important fungal pathogens. Hence, it might be concluded that bioactive compound sugiol present in *M. glyptostroboides* could serve as an effective alternative to support the antifungal activity of antimycotics. Further in vivo studies are requested to confirm the clinical efficacy of sugiol.

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References


