

Original Research Article

Effects of mycelial extract and crude protein of the medicinal mushroom, *Ophiocordyceps sobolifera*, on the pathogenic fungus, *Candida albicans*

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Abstract

Purpose: To investigate the antifungal effect of the mycelial extracts and crude proteins of the medicinal mushroom, *Ophiocordyceps sobolifera* on *Candida albicans*.

Methods: The antifungal activities of the mycelial extracts and crude proteins of seven strains of the entomopathogenic fungus, *Ophiocordyceps sobolifera*, were screened against *Candida albicans* strain NCYC854 using an agar well diffusion method. Minimum inhibitory concentrations (MIC) and minimum fungicidal concentration (MFC) were determined using broth microdilution method. The kinetics of fungal death was elucidated via time-kill assays, and while ultrastructural alteration changes to fungal cells were investigated by scanning electron microscopy (SEM).

Results: The antifungal activities of the mycelial extracts were superior to those of the crude proteins. Among the isolates, Cod-KK1643 exhibited the highest activity with the lowest MIC against the test strains. Therefore, it was chosen for further investigations. The isolate Cod-KK1643 exhibited concentration- and time-dependent fungistatic activity in the time kill assay. However, these activities were absent in the crude protein of the isolate. Moreover, Cod-KK1643 mycelial extract induced morphological alterations in fungal cells, such as decreased cell size, and crushed or cracked appearance. Slight alterations in cell morphology (decreased cell size or crushed appearance) were observed in the crude protein treatment.

Conclusion: The mycelial extracts of the fungus, *O. sobolifera* (especially isolate Cod-KK1643) exert potent antifungal activity against human pathogenic fungus, *C. albicans*.

Keywords: Anti-fungal activity, *Candida albicans*, Entomopathogenic fungus, *Ophiocordyceps sobolifera*

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INTRODUCTION

Candida albicans is the medically important fungal pathogen which asymptotically colonizes in several part of human body, but it

can overgrow and cause infection in low immunity hosts [1]. The infections due to this species range from superficial, mucosal and dermal infections, to hematogenously disseminated infections [2]. Nowadays, the

emergence and spread of antimicrobial resistance of *C. albicans* through production biofilms has been reported [1,3-5]. Therefore, to overcome the problem of this resistance, alternative sources of new and improved drugs or bioactive compounds are being screened for possible development as new antimicrobial agents.

One of the possible candidates is the entomopathogenic fungi in the genus *Cordyceps* and *Ophiocordyceps* which possess antifungal activities and produce various important bioactive compounds [6,7]. It has been reported that an anti-microbial peptide present in *Cordyceps militaris* inhibited the growth of fungal pathogens *C. albicans* and *Fusarium oxysporum* [6,7]. In addition, a bioactive compound cordytropolone isolated from *Ophiocordyceps sobolifera* inhibited the mycelial growth of the fungal pathogen *Colletotrichum* spp. under *in vitro* and *in vivo* conditions [8].

Among the entomopathogenic fungi, *O. sobolifera* has been shown to exhibit concentration- and time-dependent antibacterial activity against the bacterial pathogen *Staphylococcus aureus*, and against breast cancer MCF-7 cell line [9]. Moreover, the antimicrobial activities against the plant pathogenic fungi *Colletotrichum* spp. have also been studied and reported [8,10]. However, the medicinal value of *O. sobolifera* against the human fungal pathogen *C. albicans* has yet been elucidated. Therefore, this study was aimed at screening the effects of mycelial extract of *O. sobolifera* and crude protein against fungal pathogen *C. albicans*, using the agar well diffusion method. MICs and MFCs were determined, and the kinetics of fungal death was investigated using time-kill method. The mechanism of action was also investigated using SEM.

EXPERIMENTAL

Mycelial extract and crude protein preparation

The mycelial extracts of seven strains of *Ophiocordyceps sobolifera* (Table 1) were prepared using the method as described by Sangdee et al [11]. Briefly, the fungal mycelia of all the isolates were cultured in an induced medium [12] at 28 °C for 20 days. The mycelia were then harvested, dried and powdered. Subsequently, 100 mg/mL of each dry mycelium was prepared in 50% (v/v) ethanol. Then, the mixtures were sonicated, centrifuged and filtered through a 0.2-µm filter. The resultant extracts were used for investigation of antifungal activity.

For crude protein preparation, 3 mL of the 50 % (v/v) ethanol extract were pipetted into 15-mL Focal tubes. The crude protein was precipitated using three volumes of protein precipitation buffer. The extract and buffer were mixed by inverting 20 times before being incubated at -20 °C for 2 h. Thereafter, the mixture was centrifuged at 9100 × g for 20 min. The total proteins pellets from all the extracts were separately dissolved in 500 µL of 1 × PBS, quantized, and the final protein concentrations were adjusted to 5 mg/mL before being used for investigating antifungal activity.

Evaluation of antifungal activity of mycelial extract and crude protein

The pathogenic fungus *Candida albicans* NCYC854 was cultured on Mueller Hinton Agar (MHA) at 37 °C for 16 - 18 h. Then, one loopful of fungal pathogen was inoculated into Mueller Hinton broth (MHB) at 37 °C for 4 h with shaking at 250 rpm before being adjusted to 4-5 × 10⁶ CFU/mL. A sterile cotton swab was dipped into the standardized fungal suspension and then swabbed in four directions over the entire surface of the MHA plates. The plates were cut using a 7 mm sterile cork borer. The mycelial extracts (100 mg/mL) and crude protein (5 mg/mL) were added to each well (both at 0.1 mL per well). The plates were incubated at 37 °C for 16 - 18 h, after which the zone of inhibition surrounding in each well was measured. The negative controls used were 50% (v/v) ethanol and 1 × PBS, amphotericin B was used as a positive control.

Determination of minimum inhibitory concentrations (MICs) and minimum fungicidal concentrations (MFCs)

The MICs and MFCs were determined using the microdilution method. One colony of the pathogenic fungus *C. albicans* NCYC854 was inoculated into MHB medium and the inoculated tubes were then incubated at 37 °C under shaking condition (250 rpm) for 4 h. Then, the fungal suspension was standardized to 4-5 × 10⁵ CFU/mL, followed by addition of 10 µL of the standardized inoculum to the wells of a 96-well polystyrene tray. Then 90 µL of each diluted mycelial extracts (100, 50, 25, 12.5, 6.25, 3.125, 1.56, 0.78, and 0.39 mg/mL) and crude protein (5, 2.5, 1.25 and 0.625 mg/mL) in MHB were separately added. An uninoculated control well and a control growth well were included in each plate. Amphotericin B (20 µg/mL) was used as the reference standard. The MIC was defined as the lowest concentration of extract that remained clear after incubation, relative to the control well. Then, the suspension in each well was streaked

on MHA. The MFC was defined as the lowest concentration of extract capable of killing the test fungus. The mycelial extract and crude protein with the best potent antifungal activities were selected for further studies with time-kill assay.

Time-kill assay

The best extract and crude protein were selected for use in investigation of antifungal activity using time-kill assay with a modified method of White *et al* [13] and Aiyegoro *et al* [14]. In this process, 50- μ L aliquots of $4-5 \times 10^5$ CFU/mL standardized fungal culture were inoculated into several MHB tubes containing 4.95 mL MHB and extract at concentrations of 2 \times , 1 \times and 0.5 \times MIC, and 0.5 mg/mL of crude protein. A test tube containing MHB and the antibiotic amphotericin B at a concentration of 20 μ g/mL, and a test tube of MHB without the extract, were included in each experiment. These were incubated at 37 $^{\circ}$ C. At time intervals of 0, 2, 4, 6, and 24 h, aliquots of fungal suspension were removed, diluted, and spread onto the surface of MHA plates. The numbers of colonies were counted after incubated at 37 $^{\circ}$ C for 24 h. The number of viable fungi in each sample was plotted over time to evaluate the killing rate. A 3 \log_{10} reduction in fungal count was considered as indicative fungicidal activity.

Investigation of the effects of mycelial extract and crude protein on fungal cell morphology

The effects of the most potent extract and crude protein on the cell morphology of the pathogenic fungus *C. albicans* NCYC854 was investigated using scanning electron microscopy. The fungal cells were inoculated on MHA and incubated at 37 $^{\circ}$ C for 16 - 18 h. One loopful of fungal cells was inoculated and re-suspended in 5 mL 1 \times PBS, and the fungal suspension was standardized to $4-5 \times 10^5$ CFU/mL. Next, 0.1 mL of the standardized suspension was spread on MHA plates and incubated at 37 $^{\circ}$ C for 6 h. Then, 1 mL of various concentrations of the mycelial extract (2 \times MIC, 1 \times MIC and 0.5 \times MIC); 0.5 mg/mL of crude protein, and the antibiotic amphotericin B at a concentration of 20 μ g/mL were added. The mixtures were incubated at 37 $^{\circ}$ C for 16 - 18 h. Thereafter, the fungal cells were harvested and washed 3 times with 1 \times PBS, and fixed with 2.5 % glutaraldehyde in 5% sucrose overnight at 4 $^{\circ}$ C. Then, the fungal cell pellets were dehydrated in gradient of alcohol concentrations before being applied to a membrane. Dried samples were sputter-coated with gold before being examined under scanning electron microscope (SEM).

Statistical analysis

T-test was used for data analysis. Differences between control and treatment groups were analyzed using an analysis of variance, and means were compared by DMRT test (at $p = 0.05$). The statistical analyses were performed using SPSS version 14 (SPSS Inc., IL, USA).

RESULTS

Activity of mycelial extract and crude protein against *C. albicans*

The mycelial extracts inhibited the growth of *C. albicans* NCYC854 more than those of crude proteins. Interestingly, the inhibition zones observed for these mycelial extracts (> 15 mm) were equal to the inhibition zones produced by the control antibiotics amphotericin B at the tested concentration (Table 1). Small inhibition zones were observed for crude protein treatment of only four *O. sobolifera* isolates i.e. Cod-KK1643, Cod-KS1601, Cod-SN1606 and Cod-SN1610. These results are shown in Table 1.

Table 1: Effect of the mycelial extracts and crude proteins of *O. sobolifera* strains on *C. albicans* NCYC854

<i>O. sobolifera</i> strain	Zone of inhibition (mm)	
	Mycelial extract	Crude protein
Cod-NB1302	15.33 \pm 0.33 ^b	0.00 \pm 0.00 ^a
Cod-KK1506	16.00 \pm 1.00 ^b	0.00 \pm 0.00 ^a
Cod-KK1643	15.00 \pm 0.00 ^b	9.00 \pm 0.00 ^c
Cod-KS1601	15.33 \pm 0.33 ^b	3.00 \pm 3.00 ^{ab}
Cod-SN1606	20.33 \pm 1.20 ^c	6.00 \pm 3.00 ^{bc}
Cod-SN1610	15.00 \pm 1.00 ^b	5.33 \pm 2.66 ^{bc}
Cod-SN1626	14.66 \pm 0.33 ^b	0.00 \pm 0.00 ^a
Amphotericin B	15.00 \pm 0.00 ^b	15.00 \pm 0.00 ^d
50% ethanol	0.00 \pm 0.00 ^a	0.00 \pm 0.00 ^a
1 \times PBS	0.00 \pm 0.00 ^a	0.00 \pm 0.00 ^a

*Different lower case letters in the same column denote significant differences ($p < 0.05$)

Minimum inhibitory concentrations (MICs) and minimum fungicidal concentrations (MFCs)

The results showed that the MICs of the mycelial extracts were quite low, ranging from 0.78-25.00 mg/mL (Table 2). Out of the seven mycelial extracts, Cod-KK1643 exhibited the most potent activity, with MIC values of 0.78 mg/mL against *C. albicans* NCYC854. The MICs of crude proteins of all test isolates were more than 0.5 mg/mL. A similar trend of results with low MFC values was also observed. The mycelial extract

of Cod-KS1601 had the lowest MFC value (3.12 mg/mL) against *C. albicans* NCYC854, followed by Cod-SN1606 (6.25 mg/mL) and Cod-KK1643 (12.50 mg/mL). The MFC values of the remaining extracts were 50.0 mg/mL (Table 2). The MFCs of crude proteins of all test isolates were more than 0.5 mg/mL. Based on these results, Cod-KK1643 isolate was selected for further evaluation of antifungal activity.

Time-kill

The fungistatic or fungicidal activity of the mycelial extract and crude protein of the isolate Cod-KK1643 against *C. albicans* NCYC854 were performed using time-kill assays. The time kill profiles showed that the mycelial extract reduced viable cell counts of the fungal pathogen within 24 h, with lower viable fungal cell counts than those for the crude protein treatment (Figure 1). The fungistatic activity against the test fungal strain was detected when the concentration and exposure time of the mycelial extract were increased to 2 × MIC and 24 h, respectively, indicating that the mycelial extract exerted a concentration- and a time-dependent fungistatic activity against this fungal strain. No fungistatic or fungicidal activity was detected in a crude protein treatment (Figure 1). No viable cells were detected after 2 h exposure to the antibiotic amphotericin B (Figure 1), indicating the antibiotic is fungicidal against this fungal strain.

Effect of mycelial extract on fungal cell morphology

The effects of the mycelial extract and crude protein of the isolate Cod-KK1643 on the shape and surface characteristics of *C. albicans* NCYC854 were determined using SEM. The results showed that treatment of the *C. albicans* NCYC854 strain with the mycelial extract induced several alterations. After treatment, fungal cells decreased in size, and became crushed or cracked in appearance (Figure 2 C, D and E). Increasing the concentration of the

mycelial extract to 1 × MIC and 2 × MIC resulted in enhanced cell decreases and cracked appearance (Figure 2 D and E). Treatment with crude protein resulted in decreases in cell size or crushed appearance of the cells (Figure 2 F). Treatment with the antibiotic amphotericin B induced several alterations such as decreased cell size or crushed appearance of cell or bloated cells (Figure 2 B). The untreated control cells did not show any shape or surface alterations (Figure 2 A).

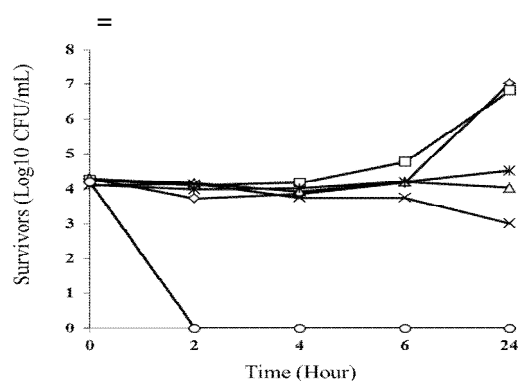


Figure 1: *In vitro* fungistatic activity of Cod-KK1643 crude protein at the concentration of 0.5 mg/mL (∗), and mycelial extracts at concentrations of 0.5 × MIC (□), 1 × MIC (△), and 2 × MIC (×), compared to an untreated control (◇) and amphotericin B at concentration of 20 µg/mL (○) against *Candida albicans* NCYC854

DISCUSSION

Ophiocordyceps sobolifera, is one of an entomopathogenic fungus which have been recently used in traditional Chinese medicine. It has produced a variety of bioactive compounds with anti-tumor activities [15], anti-aging and anti-fatigue effects [16], kidney-protective effects [17,18] and immunity-enhancing properties [19]. However, not much was known on the effect of *O. sobolifera* on the medically important fungal pathogen *C. albicans*.

Table 2: MIC and MFC of mycelial extracts and crude proteins of *O. sobolifera* strains against *C. albicans* NCYC854

<i>O. sobolifera</i> strain	MIC (mg/mL)		MFC (mg/mL)	
	Mycelial extract	Crude protein	Mycelial extract	Crude protein
Cod-NB1302	12.5	> 0.5	50.0	> 0.5
Cod-KK1506	25.0	> 0.5	50.0	> 0.5
Cod-KK1643	0.78	> 0.5	12.5	> 0.5
Cod-KS1601	1.56	> 0.5	3.12	> 0.5
Cod-SN1606	1.56	> 0.5	6.25	> 0.5
Cod-SN1610	25.0	> 0.5	50.0	> 0.5
Cod-SN1626	6.25	> 0.5	50.0	> 0.5

MIC = minimum inhibitory concentration; MBC minimum fungicidal concentration

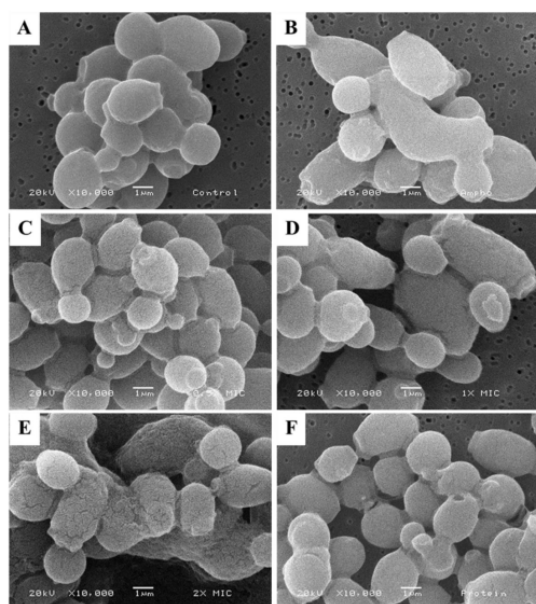


Figure 2: Scanning electron micrographs of *Candida albicans* NCYC854 after treatment with Cod-KK1643 extracts at 0.5 × MIC (C), 1 × MIC (D), 2 × MIC (E), crude protein at 0.5 mg/mL (F) and amphotericin B at 20 µg/mL (B), compared with the untreated control (A)

Therefore, in the present study, the effects of the mycelial extract and crude protein against *C. albicans* were investigated. The results demonstrated that the mycelial extract of *O. sobolifera* strains exhibited more antifungal activity than those of the crude protein extracts. For example, a large inhibition zone of > 15.00 mm against *C. albicans* was seen in the mycelial extract of the isolates Cod-KK1643 and Cod-KS1601, while a small inhibition zone (< 9.00 mm) was observed from the crude protein from the same isolates (Table 1). Moreover, the crude protein from three isolates, Cod-NB1302, Cod-KK1506 and Cod-SN1626 did not show any antifungal activity against *C. albicans*. These results are in agreement with the results from a previous study in which it was demonstrated that the mycelial extract of the medicinal fungus *Polycephalomyces nipponicus* isolate Cod-MK1201 had more antibacterial activity against human pathogenic bacteria than crude protein extract [11]. This clearly indicates that the antifungal activity of *O. sobolifera* may be due to synergistic effects of a combination of many bioactive substance(s) in the mycelial extracts, rather than the effects of crude protein extract only. This is consistent with the finding that the bioactive substances cordytropolone and adenosine from the mycelial extract of *O. sobolifera* had low antifungal activity than crude mycelial extract itself [8].

Among the fungal isolates, the Cod-KK1643 isolate had the most potent antifungal activity, as evidenced by large inhibition zones, relative to the antibiotic amphotericin B, and it had the lowest of MIC value. This result is in agreement with the finding that antifungal activity depends on the fungal strains [20]. Therefore, Cod-KK1643 was chosen for further studies on elucidation of antifungal activity with time kill assay and SEM.

The results indicate that the Cod-KK1643 mycelial extract exerted concentration- and time-dependent fungistatic activity against *C. albicans* NCYC854 strain, with induced fungal cell morphology alterations such as decreased fungal cell size and crushed/ cracked appearance (Figure 2 C - E). However, the crude protein from this extract at the test concentration, 0.5 mg/mL, induced only slight morphological changes in cell morphology (decreased cell size or crushed appearance) (Figure 2 F). These results are consistent with an earlier study which reported that the protease protein from *C. militaris* exerted a dose-dependent antifungal activity against the mycelial growth of the fungal pathogen *F. oxysporum* [6]. Based on the alterations in fungal cell morphology seen in the present study, it may be reasonably suggested that the mode of antifungal action of the extract involves disruption of fungal cell wall. These findings represent the first published information on the fungal death kinetics and mechanism of action of *O. sobolifera* mycelial extract against *C. albicans*.

CONCLUSION

The findings presented in this study show that the mycelial extracts of *O. sobolifera*, (especially Cod-KK1643), have fungistatic activity against *C. albicans* through a mechanism involving the disruption of fungal cell wall. The inhibitory activity of *C. albicans* is due to the synergistic effect of a combination of bioactive substances present in the extracts. Thus, the *O. sobolifera* isolate Cod-KK1643 is a potential source of antifungal compounds.

DECLARATIONS

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Conflict of interest

No conflict of interest is associated with this work.

Author contributions

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of the article will be borne by the authors. The author Aphidech Sangdee participated in experimental setup, data collection and analysis of data, and drafting the manuscript. Kusavadee Sangdee was responsible for experimental setup and data collection. Benjaporn Buranrat and Sutthiwan Thammawat carried out analysis of data and critically reviewed the manuscript.

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