Screening of aqueous and organic extracts from a variety of fungi for their ability to antagonise the pathogenic yeast *Candida albicans*

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KEY MESSAGES

1. Aqueous extracts and ethyl acetate extracts from the fruiting bodies and mycelia of various fungi were tested for anti-*Candida albicans* activity.
2. The aqueous extracts of the fruiting bodies of two mushrooms, *Russula nigricans* and *Suillus placidus*, elicited some inhibition of *C albicans*.
3. The ethyl acetate extracts of the fruiting bodies of all mushrooms did not produce a conspicuous inhibition of *C albicans*.
4. Accumulation of the nuclear dye SYTOX green in *C albicans* cells did not occur after exposure to the *Russula nigricans* and *Suillus placidus* aqueous extracts, suggesting that inhibition of *C albicans* is not due to a membrane permeabilising effect.

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Introduction

Yeasts belonging to the genus *Candida* cause infection in susceptible patients. The increase in *Candida* infections accompanies advancements in medicine such as invasive procedures, the use of immunosuppressive drugs for organ transplants, and the frequent administration of broad-spectrum antibiotics. *C albicans* is the most common cause of candidiasis that represents the fourth most frequent nosocomial infection involving both mucosal and deep tissues, with mortality that can exceed 40%. Azoles, polyenes, and fluorinated pyrimidines are the currently available antifungal drugs. New antifungal agents are desirable. In view of the development of fungal resistance to antifungal agents,[1] a search for new ones is warranted.

Fungi produce antifungal molecules for defence against intruding fungi. The yeast *Saccharomyces boulardii* has been used to prevent and treat intestinal infections caused by bacterial pathogens. It demonstrates an antagonistic effect on filamentation, adhesion, and biofilm formation in *C albicans*. The fungi *Pichia angusta* and *Botrytis cinerea* interact with *Candida* species. Antifungal lipopeptides are produced by *Cryptosporiopsis* and *Mycogone* spp. *Aspergillus giganteus* and *A niger* also produce antifungal peptides. Antifungal cyclic peptides are produced by *Isaria felina* and *Clavariopsis aquatica*.

Defensins are defensive peptides. Plectasin, a fungal defensin, kills bacteria by binding to the bacterial cell-wall precursor Lipid II. Plant defensins can inhibit *C albicans*[2] and thus are potentially exploitable in the treatment of fungal diseases in humans.

Antifungal peptides and proteins against plant pathogenic fungi with different N-terminal sequences have been isolated in our laboratory from edible basidiomycete fungi (mushrooms). Most were prepared by aqueous extraction and inhibited mycelial growth with IC_{50} values at micromolar concentrations.

This study aimed to test the aqueous extracts and ethyl acetate extracts of a variety of fungal species for inhibitory activity against *C albicans*.

Study design and instruments

This study was conducted from December 2010 to December 2011.

Strains and growth conditions

Clinical strain 08-189073 and ATCC strain of *C albicans* were used. An isolated colony of fungi was inoculated into 5 mL of yeast nitrogen base (YNB, pH 5.5) broth (Difco Laboratories) and incubated overnight at 37°C. A 0.1 mL aliquot of this pre-culture was inoculated into 5 mL of YNB, incubated for 24 h at 37°C, and used for all experiments.

Sample preparation

Dried fruiting bodies of mushrooms were collected from local markets and Yunnan Province, China.

Mycelia of other fungi were obtained from the Department of Microbiology, China Agricultural University, Beijing, China. The collection was composed of fruiting bodies and mycelia of fungi belonging to different orders and families. To obtain aqueous extracts, the fruiting bodies or mycelia were homogenised in liquid nitrogen using a pestle. The homogenised powder was soaked in distilled water for 12 hours and centrifuged. The resulting supernatants were concentrated by freeze-drying. To obtain organic extracts of the fungi, the powder obtained above was extracted with ethyl acetate (1 g/5 mL) for 3 hours, with changes of ethyl acetate every half hour. The ethyl acetate was removed using a rotary evaporator. Organic extracts were dissolved in dimethyl sulfoxide to increase the solubility.

Assay for antifungal activity

C albicans was incubated in 10 mL nutrient broth for 12 hours at 37°C. The suspension (5 mL) was transferred to 50 mL nutrient broth and incubated for 6 h to shift growth to the mid-logarithmic phase. The yeast suspension was then centrifuged (2000 g, 10 min). The pellet was collected and resuspended in phosphate buffer saline. Each sample was prepared in triplicate; one aliquot of yeast was mixed with the test samples at 4, 2, 1, 0.5, and 0.25 mg/mL; one aliquot of yeast was mixed with culture medium as a negative control. The mixtures were incubated in a shaker and aliquots obtained at 0, 3, 6, and 12 h, then serially diluted with nutrient broth and spread on agar plates. After incubation at 37°C for 24 h, the colonies were counted. The average number of colonies for each condition and dilution was derived from the three plates. Amphotericin B was used as positive control.

Membrane permeabilisation

This assay is based on uptake of SYTOX Green. After incubation with the nuclear dye for 10 min, fungal cells were analysed with a fluorescence microscope for internalised dye. Controls were treated with amphotericin B.

Results

Antifungal activity

The results of the assay for the aqueous extracts and ethyl acetate extracts of various fungi for activity against *C albicans* (ATCC 90028) are shown in the Table. All aqueous extracts except those of *R nigricans* and *S placidus*, and all ethyl acetate extracts brought about 2% to 12% inhibition of *C albicans* reference strain ATCC 90028 (after treatment for 12 hours at 37°C) and thus were regarded as inactive for practical purposes. The aqueous extract of *R nigricans* showed 22-40% inhibition while the aqueous extract of *S placidus* showed 11-23% inhibition at the concentrations of 1-3 mg/mL. The aqueous extract of *R nigricans* (3 mg/mL) inhibited *C albicans* reference strain ATCC 90028 and clinical strain 08-189073 by about 60% after treatment for 12 hours at 37°C.

SYTOX green uptake

SYTOX green accumulation in *C albicans* cells was not observed after exposure to *R nigricans* aqueous extract.

Discussion

In this study, a number of fungal species belonging to different orders and families were examined for their ability to inhibit *C albicans*. The inhibition elicited by the vast majority of these fungal species was too low (<20%) to be considered significant. The aqueous extracts of two fungal species, *S placidus* and *R nigricans*, elicited >20% inhibition at a concentration of 4 mg/mL and 1 mg/mL, respectively. We focused on the aqueous extract of *R nigricans* in view of its higher anti-*C albicans* activity.

The lack of SYTOX green accumulation in *C albicans* cells following treatment with *R nigricans* aqueous extract suggests that the fungal extract does not adversely affect the permeability of *C albicans* membrane and that it inhibits *C albicans* by some other mechanism. This observation is in contrast to the findings that some antifungal peptides adversely affect permeability of the *C albicans* membrane.[3]

The presence of antifungal protein previously reported in some of the fungal species examined in the present investigation, which did not inhibit *C albicans*, suggests that the isolated antifungal proteins do not inhibit *C albicans* although they are active against other fungal species. Previously we have found that some antifungal proteins exhibit broad-spectrum activity against various fungal species but other antifungal proteins demonstrate activity against only one or two fungal species.[4]

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TABLE. Anti-*Candida* activity of fungal extracts toward reference strain ATCC 90028 (treatment for 12 hours at 37°C)

Scientific Name	Mean±SD % growth inhibition of *Candida albicans*					
	Aqueous extract (mg/mL)			Ethyl acetate extract (mg/mL)		
	1	2	3	1	2	3
Fruiting bodies of fungi						
Agaricus blazei	5±0.2	4±0.4	5±0.5	-	5±0.2	5±0.2
Agrocybe cylindracea	3±0.2	6±0.2	8±0.5	5±0.1	3±0.2	8±0.8
Amanita caesarea	4±0.4	5±0.7	11±0.7	-	-	-
Amanita manginiana	4±0.2	5±0.2	4±0.4	-	4±0.2	4±0.5
Boletus bicolour	5±0.3	7±0.7	9±0.5	4±0.3	5±0.3	8±0.5
Boletus edulis	6±0.4	8±1.2	8±0.5	-	6±0.7	8±0.3
Boletus sp.	2±0.1	-	-	2±0.1	3±0.1	5±0.5
Catathelasma ventricosum	-	5±0.4	8±0.5	-	-	5±0.4
Coprinus comatus	7±0.2	6±0.5	12±1.5	2±0.6	4±0.2	2±0.5
Cortinarius collinitus	4±0.8	-	3± 0.5	4±0.1	4±0.6	8±1.8
Cortinarius lilacinus	-	6±0.3	9±0.5	-	-	5±0.5
Dictyophora duplicata	7±0.6	7±0.5	11±0.7	7±0.6	7±0.6	11±2.5
Diehiomyces microsporus	5±0.4	6±0.6	10±0.8	-	5±0.4	10±1.5
Flammulina velutipes	3±0.3	8±0.7	9±0.5	-	3±0.3	9±0.5
Gloeostereum incarnatum	-	9±0.8	11±1.0	-	-	7±0.5
Gomphidius viscidus	6±0.6	7±0.2	7±0.5	6±0.6	6±0.6	7±0.4
Hericium erinaceum	7±0.3	6±0.5	9±0.8	7±0.3	7±0.3	9±0.2
Hypsizygus marmoreus	8±0.6	7±0.6	8±0.6	5±0.6	4±0.6	4±0.6
Lactarius volemus	9±0.4	8±0.3	8±0.5	-	3±0.4	8±0.5
Lentinula edodes	7±0.5	9±0.8	8±0.5	7±0.5	7±0.5	11±0.5
Marasmius oreades	-	5±0.3	7±0.5	-	-	9±0.5
Phellinus igniarius	5±0.4	6±0.2	6±0.5	5±0.4	5±0.4	8±0.4
Pholiota nameka	6±0.1	7±0.5	12±0.9	-	6±0.1	10±0.8
Pleurotus citrinopileatus	5±0.2	6±0.6	7±0.5	5±0.2	4±0.4	6±0.5
Pleurotus eryngii	6±0.3	6±0.7	5±0.5	-	6±0.6	5±0.2
Pleurotus ostreatus	7±0.6	6±0.3	6±0.5	-	7±0.6	8±0.5
Pycnoporus sanguineus	-	-	3±0.5	6±0.3	6±0.3	10±0.7
Russula lepida	8±0.7	8±1.1	11±0.5	8±0.7	8±0.7	11±0.8
Russula nigricans	**22±4.6**	**38±3.4**	**40±3.8**	**2±0.6**	**-**	**-**
Sarcodon aspratus	6±0.3	8±0.2	6±0.5	-	-	-
Suillus pictus	7±0.2	7±0.5	11±0.5	3±0.2	6±0.4	9±0.5
Suillus placidus	**11±2.0**	**16±3.4**	**23±2.5**	**-**	**1±0.2**	**3±0.3**
Thelephora ganbajun	5±0.4	6±0.2	9±0.5	5±0.3	5±0.2	6±0.2
Trametes versicolor	6±0.2	7±0.6	10±0.5	-	6±0.2	11±0.6
Tylopilus virens	6±0.4	7±0.4	9±0.5	6±0.3	6±0.4	6±0.8
Volvariella volvacea	6±0.1	8±0.6	9±0.5	-	-	2±0.5
Wolfiporia cocos	-	4±0.3	7±0.5	-	4±0.2	5±0.5
Mycelia of fungi						
Botrytis cinerea	7±0.2	8±0.9	10±0.5	-	-	9±0.5
Coprinus comatus	5±0.2	7±0.6	9±0.5	-	5±0.2	9±0.4
Fusarium oxysporum	-	6±0.8	8±0.5	-	-	8±0.5
Fusarium solani	5±0.2	6±0.8	8±0.5	2±0.2	3± 0.2	3±0.5
Helminthosporium maydis	5±0.4	7±0.7	8±0.5	5±0.4	5±0.4	10±1.2
Rhizoctonia solani	-	6±0.3	9±0.5	-	-	5±0.5
Tricholoma mongolicum	6±0.7	7±0.5	9±0.5	5±0.1	6±0.1	5±0.3
Valsa mali	6±0.3	6±0.6	10±0.5	2±0.2	3±0.3	3±0.5

'-' denotes no inhibition or even stimulation of proliferation; inhibition <20% is considered too low to be significant

of fungal membranes by plant defensins inhibits fungal growth. Appl Environ Microbiol 1999;65:5451-8.
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