Original Articles

Effect of Cinnamaldehyde on Hyphal Growth of *C. albicans* Under Various Treatment Conditions

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ABSTRACT

This study investigated the effects of cinnamaldehyde in combatting the hyphal growth of *Candida albicans* under varying concentrations, treatment times, and temperatures to determine the potential benefits of applying this substance to anti-*Candida* foods or gargles. From the results of pretreatment with cinnamaldehyde against *Candida* hyphae, we found that its inhibitory activity seemed to be strengthened in parallel with prolonged pretreatment time and a rise in temperature, and that pretreatment of 2,000 µg/ml for only 1 minute significantly inhibited the hyphal growth of *C. albicans*. We also demonstrated by XTT assay that pretreatment with cinnamaldehyde affected the metabolic activity of *Candida* hyphal cells. These findings suggest that a warm drink or mouthwash containing cinnamaldehyde might be a candidate as a prophylactic or therapeutic tool against oral *Candida* infection.

Key words : *Candida albicans*, cinnamaldehyde, hyphal growth, crystal violet staining assay, XTT assay

Introduction

Candida albicans, a dimorphic fungus, is a member of the oral microbial flora in healthy human individuals, frequently and opportunistically growing excessively and becoming the causative agent of oral candidiasis^{1, 2)}. The hyphal form of *C. albicans* can invade human mucosal tissue, and exert its pathogenicity^{3, 4)}. Pathogenic symptoms of oral candidiasis accompanied by severe inflammation aggravate the quality of life of immunosuppressed individuals and elderly people^{5, 6)}.

Since spices and herbs have traditionally been used as anti-microbiological tools in daily life throughout the world, we and our colleagues have investigated their anti-*Candida* activity to develop new therapies^{7–9)}.

Previously, we reported that a cassia (*Cinnamomum cassia*) preparation inhibited hyphal

growth of *C. albicans* and exerted a therapeutic effect on a murine oral candidiasis model^{9, 10)}. Additionally, the results of gas chromatography / mass spectrometry (GC/MS) analysis suggested that cinnamaldehyde is the major component of the essential oil of cassia, and that it was responsible for the inhibitory activity in the cassia preparation⁹⁾.

In the present study, we examined the effect of cinnamaldehyde against hyphal growth of *C. albicans* under varying treatment times (from 1 to 60 minutes) and temperature conditions when applied to preparation of an anti-*Candida* food or gargle. We speculate that if an herbal material such as cassia preparation can be developed as a candy, drink, or gargle with a clinically therapeutic function against oral candidiasis, its active principle would need to display antifungal activity by limited term interaction with *C. albicans*. We also examined the metabolic activity of *Candida* cells after treatment with cinnamaldehyde.

Here we report that the inhibitory activity of cinnamaldehyde against *Candida* hyphal growth tends to be strengthened by prolonged pretreatment time and higher temperature condition, and that even a 1 minute pretreatment with this substance exerts an inhibitory activity against *Candida* cells and affects their metabolic activity.

Materials and Methods

C. albicans strain

C. albicans TIMM1768 was used in this study. The strain was grown on Sabouraud dextrose agar plate for 18h at 37°C. Cells were harvested with a microspatula and suspended in RPMI1640 medium containing 2.5% fetal calf serum (RP medium). The suspension of *C. albicans* was used as the inoculum for all subsequent experiments^{9, 11)}.

Preparation of cinnamaldehyde

Cinnamaldehyde was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) and dissolved in dimethylsulfoxide (DMSO) at 10% (w/w) prior to dilution with RP medium as described previously^{8, 9)}.

Measurement of activities of cinnamaldehyde against *Candida* hyphal growth

Inhibitory activity of cinnamaldehyde against *Candida* hyphal growth was assessed by a crystal violet (CV) staining method as described previously^{11–13)}. In brief, *Candida* cells were seeded in a 96-well flat bottom microplate at 5×10^3 cells/ml in a volume of 200 μ l RP medium and pre-incubated for 3 hours at 37°C in a 5% CO₂ atmosphere. After the microscopic observation of *Candida* growth, the medium was aspirated, and then 200 μ l of the cinnamaldehyde preparation was loaded onto the microplate and incubated at 37, 40, 42.5, and 45°C for 1, 20, 40, and 60 minutes. The conditions of pretreatment time and temperature were established on the assumption that cinnamaldehyde is used in gargles and foods such as candy, chewing gum, and spiced tea^{10, 14)}. (Since it is reported that humans perceive heat stimuli ($\geq 46^\circ\text{C}$) as unpleasant contact, temperature conditions were set at less than 45°C^{15–17)}.) The sample solution was removed and each well was washed twice with RP medium. Then 200 μ l of RP medium was loaded onto the microplate and

incubated for 14 to 15 hours at 37°C in a 5% CO₂ atmosphere.

The medium in the wells was discarded by inverting the microplate. Hyphal cells of *C. albicans* which adhered to the bottom of the wells were sterilized by treatment with 70% ethanol and stained with 0.01% CV. The microplate was washed with water and dried, and then 150 μ l of isopropanol containing 0.04N HCl and 50 μ l of 0.25% sodium dodecyl sulfate was added to the wells and mixed. The absorbance at 620 nm of quadruplicate samples was measured spectrophotometrically.

Metabolic activity measurement of *Candida* cells treated with cinnamaldehyde

Metabolic activity of *Candida* cells incubated for 1, 20, 60 minutes with varying concentrations of cinnamaldehyde was measured by XTT [2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide sodium salt] assay as described previously^{18–22)}. Briefly, *Candida* cells prepared at 5×10^5 cells/ml in RP medium were treated with cinnamaldehyde preparation using the same method as in the crystal violet assay. Two hundred μ l of XTT solution was added and incubated at 37°C for 2.5 hours. The plate was centrifuged (1,500 rpm, 1 min) and 100 μ l of supernatant in each well was placed in a new well. The absorbance at 450 nm of quadruplicate samples was measured spectrophotometrically.

Results

Inhibitory activities of cinnamaldehyde against *C. albicans* hyphal growth under the various experimental conditions

Inhibitory activities of cinnamaldehyde against growing hyphae of *C. albicans* after treatment for 20, 40, and 60 minutes at 37, 40, 42.5, 45°C were examined by CV staining method, and the results are shown in Table 1.

Inhibitory activity of each preparation was compared by IC₅₀, the concentration of cinnamaldehyde which reduced growth of *C. albicans* by 50% for the non-treatment group (control). IC₅₀ of the 20 minute pretreatment at 37°C was 320–800 μ g/ml. When pretreatment time was prolonged to 40 or 60 minutes at the same temperature, IC₅₀ for both periods was decreased to 128–320 μ g/ml. It was also shown that IC₅₀ (128–320 μ g/ml) of the 20 minute pretreatment at 40°C was lower than IC₅₀

Table 1. Inhibitory activity of cinnamaldehyde on *Candida* hyphal growth under the conditions of varying temperature and time

Treatment temperature (°C)	Treatment time (min)	IC ₅₀ (hyphal growth) (concentration: µg/ml)
37	20	320-800
	40	128-320
	60	128-320
40	20	128-320
	40	128-320
	60	51.2-128
42.5	20	128-320
	40	128-320
	60	51.2-128
45	20	128-320
	40	51.2-128
	60	51.2-128

Inhibitory activity of cinnamaldehyde against *Candida* hyphal growth was measured by crystal violet (CV) staining method as described in Materials and Methods.

Each group was treated with cinnamaldehyde for 20, 40, and 60 minutes at 37, 40, 42.5 and 45°C, respectively.

The concentration of 50% inhibition against *Candida* hyphal growth is indicated as the range between two concentrations.

Table 2. Inhibitory activity of 1 minute pretreatment with cinnamaldehyde against *Candida* hyphal growth

Treatment temperature (°C)	Relative values of <i>Candida</i> mycelia (%)	
	2,000 µg/ml of cinnamaldehyde	800 µg/ml of cinnamaldehyde
37	6.26	100
40	5.61	99.5
42.5	2.92	77.3
45	2.83	68.1

Inhibitory activity of cinnamaldehyde for *Candida* hyphal growth was measured by CV staining method as described in Materials and Methods.

Each group was treated with 2,000 or 800 µg/ml of cinnamaldehyde for 1 minute at 37, 40, 42.5 and 45°C, respectively.

Results are shown as the relative value of *Candida* hyphal growth under each pretreatment condition for non-pretreatment group (100%).

(320-800 µg/ml) at 37°C. In the 60 minute pretreatment, IC₅₀ (51.2-128 µg/ml) at 40°C was lower than IC₅₀ (128-320 µg/ml) of the same pretreatment time at 37°C. IC₅₀ at 42.5 and 45°C was at almost the same level as that of the 40 minute pretreatment, but that at 45°C (51.2-128 µg/ml) was the same as IC₅₀ of the 60 minute pretreatment at 42.5 and 45°C. These

results show that the inhibitory activity of cinnamaldehyde against *Candida* mycelial growth tends to be enhanced by prolonged pretreatment time and rising temperature.

Using the CV staining method (Table 2), we also evaluated the inhibitory activity of cinnamaldehyde against *Candida* hyphae for 1 minute to

Table 3. Metabolic activity of *Candida* hyphal cells treated with cinnamaldehyde under the conditions of varying temperature and time

Treatment temperature (°C)	Treatment time (min)	Metabolic activity of <i>C. albicans</i> (%)				
		2,000 μ g/ml of cinnamaldehyde	800 μ g/ml of cinnamaldehyde	320 μ g/ml of cinnamaldehyde	128 μ g/ml of cinnamaldehyde	control
37	1	65.7 $\pm$ 0.11	87.7 $\pm$ 3.45	85.2 $\pm$ 4.00	95.1 $\pm$ 5.07	100
	20	23.2 $\pm$ 4.60	27.1 $\pm$ 4.19	35.6 $\pm$ 4.87	72.1 $\pm$ 4.64	100
	60	10.2 $\pm$ 0.76	11.5 $\pm$ 0.59	17.6 $\pm$ 0.40	44.6 $\pm$ 3.73	100
45	1	56.0 $\pm$ 3.45*	74.5 $\pm$ 7.90*	84.8 $\pm$ 1.07	87.5 $\pm$ 4.94	100
	20	12.4 $\pm$ 0.86*	15.0 $\pm$ 2.13*	19.8 $\pm$ 2.12*	66.7 $\pm$ 6.30	100
	60	6.64 $\pm$ 0.41*	7.40 $\pm$ 0.41*	8.10 $\pm$ 0.44*	27.1 $\pm$ 1.83*	100

Metabolic activity of *Candida* hyphal cells was measured by XTT assay as described in Materials and Methods. Each group was treated with cinnamaldehyde for 1, 20, and 60 minutes at 37°C and 45°C, respectively.

Metabolic activity is shown as the relative value of each pretreatment group to the values of non-pretreatment group (control).

The values at 37°C and 45°C were compared by corresponding values of each treatment time and dose condition, respectively.

P values of < 0.05 were considered as significant. (* : *p* values of < 0.05)

determine its efficacy using a pretreatment time shorter than 20 minutes.

Since 1 minute pretreatment with 800 μ g/ml of cinnamaldehyde at 37°C did not inhibit hyphal growth significantly in preliminary experiments, we examined its inhibitory activity at the concentrations of 800 and 2,000 μ g/ml at 37, 40, 42.5, 45°C. The inhibitory activity of 1 minute pretreatment against *Candida* hyphal growth was shown as the relative growth of *Candida* hyphae of each pretreatment group to the non-pretreatment group (control). While the pretreatment with 800 μ g/ml cinnamaldehyde for 1 minute at 37°C did not inhibit the hyphal growth of *C. albicans*, pretreatment with the same dose at 45°C lowered the growth of these hyphae to 68.1%.

With 2,000 μ g/ml pretreatment, *Candida* hyphal growth was lowered to 6.26% at 37°C; further, growth of these hyphae pretreated with the same amount of material at 45°C was inhibited to 2.83%.

These data indicate not only that the inhibitory activity of cinnamaldehyde seemed to be strengthened by prolonged pretreatment time and rising temperature, but also that 2,000 μ g/ml of this substance strongly inhibited hyphal growth of *C. albicans*, when preincubated with it even for 1 minute.

Metabolic activity of *Candida* cells treated with cinnamaldehyde

Effects of pretreatment with cinnamaldehyde on the metabolic activity of *C. albicans* hyphal cells were also examined by XTT assay.

Table 3 shows that metabolic activity of these cells decreased from 95.1% (128 μ g/ml, 1 minute, 37°C) to 6.64% (2,000 μ g/ml, 60 minutes, 45°C), and tended to decrease in a time- or dose-dependent manner at 37°C and 45°C. Comparing the metabolic activity at 37 and 45°C, the hyphae treated at 45°C seemed to be lower than the corresponding values at 37°C.

The results also show that metabolic activity of *Candida* cells treated with 2000 μ g/ml cinnamaldehyde for 1 minute decreased to 65.7% at 37°C, and the hyphal growth after 1 minute pretreatment at 45°C (56.0%) was lower than the corresponding value at 37°C. The results of XTT assay demonstrate that even 1 minute pretreatment of cinnamaldehyde lowered the metabolic activity of *Candida albicans* and this effect seemed to be stronger at 45°C.

Discussion

We examined the inhibitory activity of cinnamaldehyde pretreatment against *Candida* hyphal growth at varying times and temperatures, since

this material has been a part of the regular diet in cooked foods and teas in many countries throughout the ages²³⁾.

In this study, we demonstrated that pretreatment of *C. albicans* with cinnamaldehyde (2,000 µg/ml) for just 1 minute, exerted an inhibitory activity on its hyphal growth. The dose of 2,000 µg/ml seems somewhat higher than that usually intaken in daily life, but this concentration has been reported as safe to be consumed^{9, 10, 24)}.

The results of CV staining and XTT assay showed that the inhibitory activity of cinnamaldehyde seemed to be strengthened in parallel with a rise in temperature in warm tea which is not rejected for its unpleasant temperature effect^{16, 17)}. In the previous study, we reported that oral administration of 50 µl of a solution with 19.5 mg/ml of cinnamaldehyde or cassia which contained 19.5 mg/ml of cinnamaldehyde exerted a therapeutic activity against murine oral candidiasis^{9, 11)}. This effective concentration is about 10 times that of the 2,000 µg/ml effective *in vitro* in the pretreatment assay. We do not believe that this difference is strange because oral administration would be followed by diffusion in the oral cavity^{7, 9, 10)}. It is also our opinion that the temperature dependence on cinnamaldehyde effects *in vitro* suggest that oral intake of a hot food or beverage containing this substance such as spiced tea in daily life may be one of the more effective methods of inhibiting *Candida* hyphal growth.

It now appears to us necessary that in order to realize the successful application of cinnamaldehyde to combat human oral candidiasis, we must examine other optimal conditions, such as optimal pH, physical condition, potentiation of the effects of cinnamaldehyde in combination with other food agents and details of interaction between cinnamaldehyde and *Candida albicans*^{10, 23, 25)}. It is our hope that these studies will facilitate development of the application of spices and herbs in daily use to prevent and improve the problem of oral candidiasis in elderly and immunocompromised patients.

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