Original Articles

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

Yuuki Taguchi¹, Yayoi Hasumi², Kazumi Hayama¹, Ryo Arai¹, Yayoi Nishiyama², Shigeru Abe²

¹ Research and Product Development Division, S&B Foods Inc.
² Teikyo University Research Institute of Medical Mycology

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 candidiasis¹-². The hyphal form of *C. albicans* can invade human mucosal tissue, and exert its pathogenicity³-⁴. Pathogenic symptoms of oral candidiasis accompanied by severe inflammation aggravate the quality of life of immunosuppressed individuals and elderly people⁵-⁶.

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 therapies¹-².⁶-⁹.

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 model¹⁰. 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 preparation⁶.

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 37ºC. 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.

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. Cinnamaldehyde was prepared at 5 × 10⁵ cells/ml in RP medium and treated with cinnamaldehyde preparation using the same method as in the crystal violet assay. Two hundred µl of XTT solution was added and incubated at 37ºC for 2.5 hours. The plate was centrifuged (1,500 rpm, 1 min) and 100 µl 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 Candida 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, 45ºC were examined by CV staining method, and the results are shown in Table 1. Inhibitory activity of each preparation was compared by IC₅₀, which was defined as the concentration of cinnamaldehyde that reduced growth of C. albicans by 50% for the non-treatment group (control). IC₅₀ of the 20 minute pretreatment at 37ºC was 320-800 µg/ml. When pretreatment time was prolonged to 40 or 60 minutes at the same temperature, IC₅₀ for both periods was decreased to 128-320 µg/ml. It was also shown that IC₅₀ (128-320 µg/ml) of the 20 minute pretreatment at 40ºC was lower than IC₅₀...
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 1. Inhibitory activity of cinnamaldehyde on Candida hyphal growth under the conditions of varying temperature and time

<table>
<thead>
<tr>
<th>Treatment temperature (°C)</th>
<th>Treatment time (min)</th>
<th>IC₅₀ (hyphal growth) (concentration: µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>20</td>
<td>320-800</td>
</tr>
<tr>
<td>37</td>
<td>40</td>
<td>128-320</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>128-320</td>
</tr>
<tr>
<td>40</td>
<td>20</td>
<td>128-320</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>128-320</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>51.2-128</td>
</tr>
<tr>
<td>42.5</td>
<td>20</td>
<td>128-320</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>128-320</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>51.2-128</td>
</tr>
<tr>
<td>45</td>
<td>20</td>
<td>128-320</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>51.2-128</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>51.2-128</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Treatment temperature (°C)</th>
<th>Relative values of Candida mycelia (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2,000µg/ml of cinnamaldehyde</td>
</tr>
<tr>
<td>37</td>
<td>6.26</td>
</tr>
<tr>
<td>40</td>
<td>5.61</td>
</tr>
<tr>
<td>42.5</td>
<td>2.92</td>
</tr>
<tr>
<td>45</td>
<td>2.83</td>
</tr>
</tbody>
</table>

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 800µg/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%).

(320-800µg/ml) at 37°C. In the 60 minute pretreatment, IC₅₀ (51.2-128µg/ml) at 40°C was lower than IC₅₀ (128-320µg/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 (51.2-128µg/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
determine its efficacy using a pretreatment time shorter than 20 minutes.

Since 1 minute pretreatment with 800 µg/ml of cinnamaldehyde at 37ºC did not inhibit hyphal growth significantly in preliminary experiments, we examined its inhibitory activity at the concentrations of 800 and 2,000 µg/ml at 37, 40, 42.5, 45ºC. 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 µg/ml cinnamaldehyde for 1 minute at 37ºC did not inhibit the hyphal growth of C. albicans, pretreatment with the same dose at 45ºC lowered the growth of these hyphae to 68.1%.

With 2,000 µg/ml pretreatment, Candida hyphal growth was lowered to 6.28% at 37ºC; further, growth of these hyphae pretreated with the same amount of material at 45ºC 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 µg/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 µg/ml, 1 minute, 37ºC) to 6.64% (2,000 µg/ml, 60 minutes, 45ºC), and tended to decrease in a time- or dose-dependent manner at 37ºC and 45ºC. Comparing the metabolic activity at 37 and 45ºC, the hyphae treated at 45ºC seemed to be lower than the corresponding values at 37ºC.

The results also show that metabolic activity of Candida cells treated with 2000 µg/ml cinnamaldehyde for 1 minute decreased to 65.7% at 37ºC, and the hyphal growth after 1 minute pretreatment at 45ºC (56.0%) was lower than the corresponding value at 37ºC. 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 45ºC.

**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 ages\textsuperscript{22}.

In this study, we demonstrated that pretreatment of \textit{C. albicans} with cinnamaldehyde (2,000 \(\mu g/ml\)) for just 1 minute, exerted an inhibitory activity on its hyphal growth. The dose of 2,000 \(\mu g/ml\) seems somewhat higher than that usually taken in daily life, but this concentration has been reported as safe to be consumed\textsuperscript{3,10,24}. 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\textsuperscript{10,17}. In the previous study, we reported that oral administration of 50\(\mu l\) 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\textsuperscript{7,9,10}. This effective concentration is about 10 times that of the 2,000\(\mu g/ml\) effective \textit{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\textsuperscript{7,9,10}. It is also our opinion that the temperature dependence on cinnamaldehyde effects \textit{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 \textit{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 \textit{Candida albicans}\textsuperscript{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 immuno-compromised patients.

References

14) Zhu M, Carvalho R, Scher A, Wu CD: Short-term germ-killing effect of sugar-sweetened cinnamon chewing gum on salivary anaerobes associated

\textbf{Med. Mycol. J. Vol. 53 (No. 3), 2012 203}
22) Knight SA, Dancis A: Reduction of 2, 3-bis (2-methoxy-4-nitro-5-sulphophenyl) −2H-tetrazolium-5-carboxanilide inner salt (XTT) is dependent on CaFRE10 ferric reductase for Candida albicans grown in unbuffered media. Microbiology 152: 2301-2308, 2006.