

ANTIFUNGAL ACTIVITY OF CINNAMALDEHYDE AGAINST *CANDIDA ALBICANS*

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Abstracts. *Candida albicans* is a common pathogen, especially among immunocompromised patients. It is beginning to show resistance against the azole drug group, which is usually used to treat this pathogen. We studied the antifungal effects of cinnamaldehyde against *C. albicans*. Germ tube formation of *C. albicans* exposed to cinnamaldehyde was determined by the crystal violet based method. The effect of cinnamaldehyde on adhesion of *C. albicans* to buccal epithelial cells was investigated. Proteinase and phospholipase activities of *C. albicans* in the presence of cinnamaldehyde were assessed using bovine serum albumin agar and egg yolk agar, respectively. In this study, cinnamaldehyde possessed antifungal activity against *C. albicans* with a minimum inhibitory concentration of 125 µg/ml. At sub-inhibitory concentrations, cinnamaldehyde significantly reduced germ tube formation, proteinase and phospholipase activities in a dose dependent manner ($p < 0.01$). Cinnamaldehyde also significantly inhibited the adhesion of *C. albicans* to buccal epithelial cells ($p < 0.01$). In our study, cinnamaldehyde had *in vitro* activity against *C. albicans* and inhibited some of its virulence factors.

Keyword: *Candida albicans*, cinnamaldehyde, germ tube formation, proteinase, phospholipase

INTRODUCTION

Candida albicans is a yeast-like fungus found as normal flora on mucous membranes of healthy persons (Odds, 1987; Ganguly and Mitchell, 2011). It can also be an opportunistic pathogen causing infections ranging from superficial mucosal lesions to septicemia (Pappas *et al*, 2004). There are several putative virulence factors

of *C. albicans* which may participate in and influence the infective process, including adhesion, invasion, and the destruction of host immune factors (Odds, 1994; Hube and Naglik, 2001). Germ tubes and short hyphal elements have been shown to contribute to adherence of the organism to host epithelium, invasion and tissue damage (Jacobsen *et al*, 2012). Secretion of hydrolytic enzymes during infection is also required. Among these enzymes, secreted aspartyl proteinases (Sap), phospholipases and lipases significantly contribute to the process of nutrient acquisition, invasion, tissue damage and evasion of host responses (Ghannoum, 2000; Naglik *et al*, 2003; Schaller *et al*, 2005; Gácsér *et al*, 2007).

Candidiasis can cause morbidity and

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mortality. Reasons for this include limited numbers of antifungal drugs and widespread use of these drugs for treatment, leading to development of drug-resistance (White *et al*, 1998). Thus, the search for new and effective products with antifungal activity against *C. albicans* is needed. Previous studies have reported cinnamaldehyde, a major active ingredient in cinnamon, to have antimicrobial activity against bacteria (Ooi *et al*, 2006), viruses (Hayashi *et al*, 2007) and fungi, including *C. albicans* (Ooi *et al*, 2006; Shreaz *et al*, 2011a). In this study, we evaluated the antifungal activity of cinnamaldehyde against the growth and virulence factors of *C. albicans*.

MATERIALS AND METHODS

C. albicans strains

Twenty clinical isolates of *C. albicans* obtained from blood cultures and two reference stains (ATCC 10231 and 90028) were used. The strains were cultured on Sabouraud dextrose agar (SDA) (Oxoid®, Hamshire, UK) and incubated at 37°C for 48 hours before use.

Determination of anti-candidal activity of cinnamaldehyde by agar disc diffusion

The antifungal activity of cinnamaldehyde (Sigma, St Louis, MO) against *C. albicans* was tested by disc diffusion assay (Hammer *et al*, 1998). Yeast suspension was prepared in sterile 0.85% normal saline solution (NSS) and the density was adjusted to a turbidity of a 0.5 McFarland standard (1×10^6 to 5.0×10^6 CFU/ml). The inoculum was swabbed in three directions on SDA. These inoculated plates were left to dry for 5 minutes. A sterilized filter paper disc (6 mm diameter) was placed on the agar and 5 µl of cinnamaldehyde (1 mg/µl) was spotted onto the disc. The plate was incubated at 37°C for 48 hours. The diameters (mm) of inhibition zone were determined.

Determination of minimum inhibitory concentration and minimum fungicidal concentration of cinnamaldehyde

The minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) of cinnamaldehyde against *C. albicans* strains were determined using the method of Khan *et al* (2013) with some modification. Briefly, cinnamaldehyde was serially diluted in Sabouraud dextrose broth (Oxoid®) with 0.02% Tween-80 (SDB-T) to achieve a range of concentration of 195-1,000 µg/ml and an equal volume of yeast suspension (1×10^4 CFU/ml) was added. The suspensions were incubated at 37°C for 48 hours. The MIC was determined visually and defined as the lowest concentration of the cinnamaldehyde that inhibited growth of yeast cells, as indicated by the absence of turbidity. Sub-culturing the optically clear tubes was performed to determine the MFC. The experiments were repeated in triplicate.

Time-kill analysis of cinnamaldehyde

The time-kill study of cinnamaldehyde against *C. albicans* was performed using the method of Cantón *et al* (2004) with some modification. Briefly, *C. albicans* ATCC 10231 was added to SDB-T containing an appropriate concentrations of cinnamaldehyde to give a final concentration of 5×10^4 CFU/ml; the initial concentrations of cinnamaldehyde were 0, 1/4, 1/2 and 1X MIC. The suspension was incubated at 37°C and the viability of the yeast was determined at 0, 1, 2, 4, 6, 12, 24 and 48 hours using the colony count method. The experiments were repeated in duplicate.

Anti-candidal activity of cinnamaldehyde on germ tube formation of *C. albicans* by crystal violet based technique

To determine the effect of cinnamaldehyde on germ tube formation, a cell

suspension (1×10^6 CFU/ml) of *C. albicans* was prepared and 0.1 ml was added to the wells of 96-flat-microtiter plates containing 10% fetal bovine serum (Gibco BRL, Gaithersburg, MD) and cinnamaldehyde at final concentrations of 0 (control), 1/4, and 1/2XMIC. Wells without yeast cells were set up as blanks. After incubation at 37°C for 2 hours, the plates were processed as described elsewhere (Abe *et al*, 1994). Briefly, the medium in the wells were discarded and washed once with 100 μ l of 70% ethanol. Yeast cells without germ tubes were removed by washing once with 200 μ l 0.25% sodium dodecyl sulfate (SDS) (Ajax Finechem, Taren Point, NSW, Australia) and three times with distilled water. Germ tube forming cells attached to the wells were stained with 100 μ l 0.01% crystal violet (Sigma, St Louis, MO) for 15 minutes. The dye solution was removed and the plate was washed three times with water, once with 0.25% SDS and twice more with water. After the plate dried, 200 μ l of isopropanol containing 0.04 N HCl, and 50 μ l of 0.25% SDS was added to each well and mixed briefly. The absorbance at 570 nm was determined using an ELISA reader (Dynex, Chantilly, VA). The experiments were repeated in triplicate.

Buccal epithelial cell adhesion inhibition activity of cinnamaldehyde

Buccal epithelial cell (BEC) adhesion of *C. albicans* in the presence of cinnamaldehyde was tested as previously described (Taweekhaisupapong *et al*, 2005) with minor modification. *C. albicans* ATCC 10231 was used in this study. Briefly, yeast cells (5×10^5 CFU/ml) were incubated in SDB-T containing cinnamaldehyde at different concentrations at 37°C for 1 hour. Treated cells were washed two times and resuspended in NSS to give a concentration of 1×10^7 CFU/ml. BECs were collected from three healthy human subjects by gently

rubbing the inside of the cheeks with sterile swabs and pooled in sterile NSS. The pooled BEC suspension was washed three times in NSS by centrifugation at 3,000g for 15 minutes to eliminate debris and loosely attached microorganisms. The BECs were then re-suspended in NSS to a concentration of 1×10^5 CFU/ml. Then, 100 μ l of the solution containing BEC was added to the *C. albicans* suspension, gently mixed and incubated on a rocker (C24 Incubator Shaker; New Brunswick Scientific, Edison, NJ) at 160 rpm at 37°C for 1 hour. The cells were then filtered through a polycarbonate filter with 12 μ m pores (Millipore, Billerica, MA). The filters were then washed with 50 ml of NSS to remove unattached yeast. Each filter was carefully removed with a forceps and placed on a glass slide. The slides were air-dried, fixed with methanol and stained with Gram's stain. The number of yeast cells adhering to 100 BECs was quantified by light microscopy. The method used to quantify the number of adherent yeast was: 1) yeast with daughter cells smaller than mother cells were counted as one unit, 2) overlapping and folded BECs were excluded, and 3) only single BEC were counted. (Taweekhaisupapong *et al*, 2005) The quantification was performed in duplicate.

Anti-candidal activity of cinnamaldehyde on proteinase and phospholipase activities

The effects of cinnamaldehyde on proteinase and phospholipase activities of *C. albicans* were determined using the method adapted from Baboni *et al* (2009). A cell suspension (1×10^6 CFU/ml) was prepared and 5 μ l was spotted on bovine serum albumin agar (BSAA: 1.5% agar containing 1% yeast extract, 2% glucose, and 0.2% BSA fraction V at a pH of 4) and egg yolk agar (EYA: 1.3% Sabouraud dextrose agar containing 1.17% NaCl, 0.011%

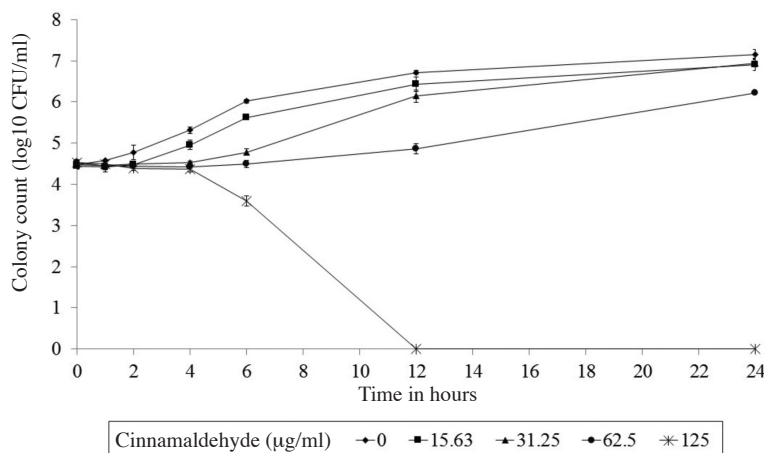


Fig 1–The killing time plot for cinnamaldehyde against *C. albicans* ATCC 10231.

CaCl₂, and 10% sterile egg yolk at a pH of 6) containing 0.02% Tween-80 with either 0 (control), 1/4 or 1/2XMIC of cinnamaldehyde. The inoculated BSAA and EYA plates were incubated in a moist chamber at 37°C for 2 and 5 days, respectively. Proteinase and phospholipase activities were then determined by the precipitation zone ration (Pz), where the Pz was the ratio of the diameter of the precipitation zone with colony to the colonies diameter. The experiment was repeated in triplicate.

Statistical analysis

Germ tube formation, BEC adhesion and proteinase and phospholipase activities of *C. albicans* in the presence of 0, 1/4 and 1/2 XMIC for cinnamaldehyde were compared using the Wilcoxon signed-rank test. A *p*-value < 0.05 was considered statistically significant.

RESULTS

Antifungal activity of cinnamaldehyde against *C. albicans*

The results of the disc diffusion method show cinnamaldehyde at 5 mg/

disc caused an inhibition zone of 60 mm. The MIC and MFC for cinnamaldehyde against *C. albicans* were the same: 125 µg/ml.

Time-kill curve for cinnamaldehyde

The results of the time-kill assay show the ability of cinnamaldehyde to kill *C. albicans* was dose dependent. At twelve hours incubation at 1XMIC for cinnamaldehyde the viability de-

creased by more than 3 to the log₁₀ (CFU/ml) or 99.9% of the initial inoculum (Fig 1).

Effect of cinnamaldehyde on germ tube formation of *C. albicans*

Germ tube formation of *C. albicans* yeast cells treated with various concentrations of cinnamaldehyde was determined by microscopy (Fig 2) and a crystal violet based method. Compared to the control, 31.25 µg/ml and 62.5 µg/ml of cinnamaldehyde reduced germ tube formation from an OD₅₇₀ of 0.45 ± 0.11 to 0.30 ± 0.09 and 0.18 ± 0.04 (*p*<0.01), respectively (Table 1). These showed the inhibitory effect of cinnamaldehyde on germ tube formation was dose-dependent.

Effect of cinnamaldehyde on BEC adhesion activity of *C. albicans* ATCC 10231

The BEC adhesion activity of *C. albicans* ATCC 10231 was significantly reduced when cinnamaldehyde was present (*p*<0.01). The average number of yeast cells adhering to the BEC decreased from 226 ± 8.49 among untreated cells to 68 ± 4.24 and 17 ± 2.12 among treated cells with cinnamaldehyde at 31.25 and 6.25 µg/ml, respectively (Table 2).

Table 1
Inhibitory effect of cinnamaldehyde on germ tube formation of *C. albicans* (n=22) determined by crystal violet stain measured at OD_{570 nm}.

Cinnamaldehyde concentration in $\mu\text{g/ml}$	Germ tube formation (OD _{570 nm})	
	Mean \pm SD	Range
0	0.45 \pm 0.11	0.25-0.62
31.25	0.30 \pm 0.09 ^a	0.14-0.44
62.5	0.18 \pm 0.04 ^a	0.11-0.25

^a $p < 0.01$; OD_{570 nm}, optical density at 570 nm; SD, standard deviation.

Table 2
Effect of cinnamaldehyde on buccal epithelial cells adhesion of *C. albicans* ATCC 10231.

Cinnamaldehyde concentration in $\mu\text{g/ml}$	Yeast cells/100 BEC	
	Mean \pm SD	Range
0	226 \pm 8.49	220-232
31.25	68 \pm 4.24 ^a	65-71
62.5	17 \pm 2.12 ^a	15-18

^a $p < 0.01$; BEC, buccal epithelial cell; SD, standard deviation.

Effect of cinnamaldehyde on proteinase and phospholipase activities

The effect of cinnamaldehyde on proteinase and phospholipase activities of *C. albicans* clinical isolates and reference strains was determined by spotting the yeast cells on BSAA and EYA containing 0, 31.25, and 62.5 $\mu\text{g/ml}$ of cinnamaldehyde (Fig 3, 4). In this study, two reference stains and fourteen clinical isolates exhibiting proteinase and phospholipase activities were used. The Pz for *C. albicans* not exposed to cinnamaldehyde for the proteinase was 2.17 ± 2.17 and for the phospholipase was 1.98 ± 0.46 . The activities of these enzymes were significantly reduced ($p < 0.01$), when the yeast was exposed to cinnamaldehyde at 31.25 and 62.5 $\mu\text{g/ml}$, to 1.92 ± 0.17 and 1.45 ± 0.27

for the proteinase and 1.65 ± 0.49 and 1.40 ± 0.38 for the phospholipase, respectively. This suggests cinnamaldehyde may reduce the activities of *C. albicans* proteinase and phospholipase in a dose dependent manner (Table 3).

DISCUSSION

Cinnamaldehyde has been previously reported to have antimicrobial activity against bacteria (Ooi *et al*, 2006), viruses (Hayashi *et al*, 2007) and fungi, including *Candida* sp (Ooi *et al*, 2006; Shreaz *et al*, 2012). In our study, both the broth dilution method and the time-kill assay gave a MIC for cinnamaldehyde against *C. albicans* of 125 $\mu\text{g/ml}$. Since many patients requiring antifungal therapy are immunocompro-

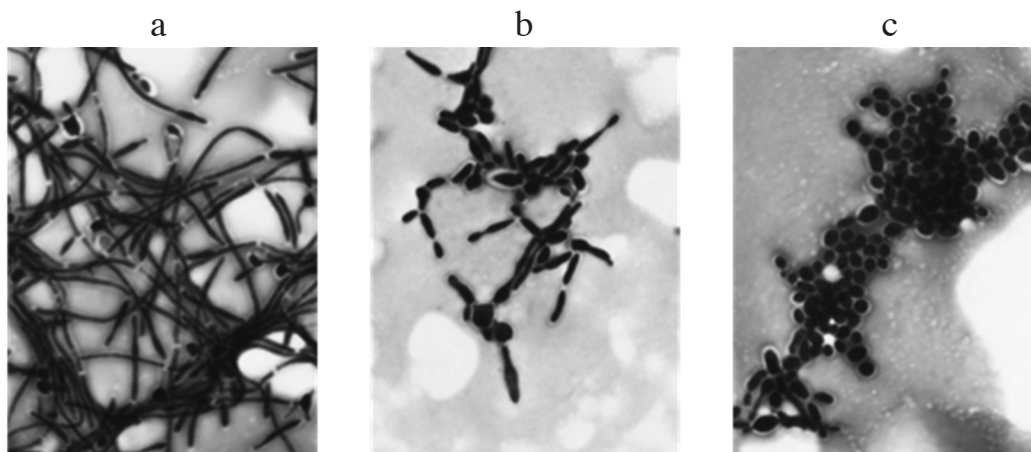


Fig 2—Germ tube formations of *C. albicans* in the presence of 0 (a), 31.25 (b) and 62.5 $\mu\text{g/ml}$ (c) of cinnamaldehyde (magnification 400 \times).

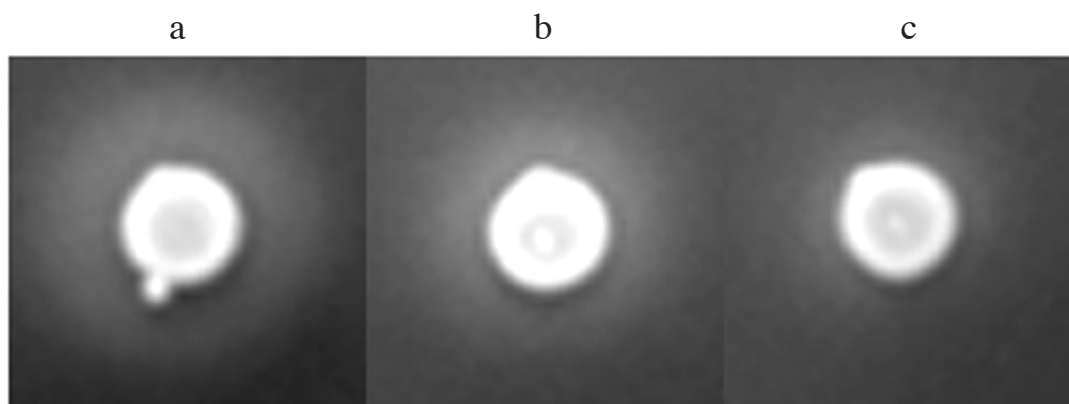


Fig 3—Culture of *C. albicans* on bovine serum albumin agar containing cinnamaldehyde at 0 (a), 31.25 (b), and 62.5 $\mu\text{g/ml}$ (c). After incubation, zone of degradation indicating proteinase activity is seen around the yeast colony. In comparison with unexposed cell (a), the clearance zone of *C. albicans* decreased with increasing cinnamaldehyde concentrations (b, c).

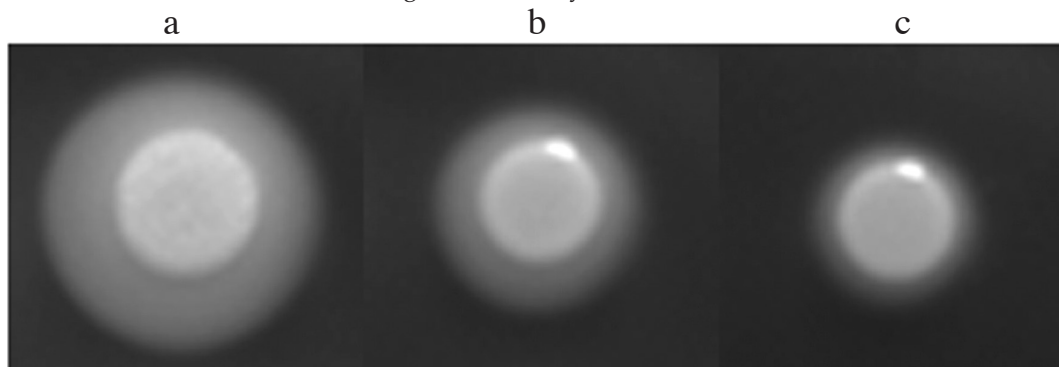


Fig 4—Culture of *C. albicans* on egg yolk agar (EYA). In the absence of cinnamaldehyde (a), the opaque precipitate zone around the yeast colony indicating phospholipase activity was clearly seen. The precipitation zone decreased when the yeast was exposed to cinnamaldehyde at 31.25 (b) and 62.5 $\mu\text{g/ml}$ (c).

Table 3

Effect of cinnamaldehyde on proteinase and phospholipase activities of *C. albicans* ($n=16$) determined with bovine serum albumin agar and egg yolk agar. The enzyme activity (Pz) was measured by dividing the diameter of the colony plus the degradation zone of proteinase or precipitation zone of phospholipase by the diameter of the colony.

Cinnamaldehyde concentration in $\mu\text{g/ml}$	Proteinase activity (cm)		Phospholipase activity (cm)	
	Mean \pm SD	Range	Mean \pm SD	Range
0	2.17 \pm 0.21	1.80-2.45	1.98 \pm 0.46	1.17-2.48
31.25	1.92 \pm 0.17 ^a	1.64-2.27	1.65 \pm 0.49 ^a	1.00-2.50
62.5	1.45 \pm 0.27 ^a	1.00-2.10	1.40 \pm 0.38 ^a	1.00-2.00

^a $p < 0.01$; cm, centimeter; SD, standard deviation.

mised, an antifungal agent with fungicidal activity is highly desirable (Wade, 1997). In our study, cinnamaldehyde appeared to have fungicidal activity *in vitro*. Mostazole drugs are fungistatic and fail to completely eliminate yeast, leading to acquired resistance of *C. albicans* to the azoles (Parks and Casey, 1996).

Germ tube formation is a pathological characteristic of *C. albicans* that facilitates invasion (Kretschmar *et al*, 1999; Gow *et al*, 2002). Previous studies have found exposure to sub-inhibitory concentrations of garlic extract, date extract and *Streblus asper* leaf-extract inhibited *Candida* germination (Ghannoum, 1990; Abu-Elteen, 2000; Taweechaisupapong *et al*, 2005). In our study, cinnamaldehyde suppressed germ tube formation at sub-inhibitory concentrations (1/4 and 1/2xMIC). Our results are supported by a previous study which found cinnamaldehyde inhibited the germination of *C. albicans* (Taguchi *et al*, 2012, 2013).

Cinnamaldehyde is a major component of cinnamon oil and has been reported to exhibit antifungal activity against *C. albicans* (Ooi *et al*, 2006). In our study, cinnamaldehyde at sub-inhibitory

concentrations reduced the proteinase and phospholipase activities of *C. albicans* ($p < 0.01$). Our findings support a previous study (Shreaz *et al*, 2012) that reported cinnamaldehyde and other cinnamic aldehydes may suppress the hydrolytic enzyme secretion of *C. albicans*. It has been purposed that cinnamaldehyde causes yeast cell membrane damage by suppressing the ergosterol biosynthetic pathway (Shreaz *et al*, 2011b; Rajput and Karuppayil, 2013) and simultaneously interacting with the membrane by binding to ergosterol (Khan *et al*, 2013). Cinnamaldehyde and its derivatives have also been reported to have a strong inhibitory effect on plasma membrane ATPase (PM-ATPase) (Shreaz *et al*, 2011b). PM-ATPase plays a regulatory role in dimorphism of *C. albicans* (Kaur and Mishra, 1991) and is involved in hydrolytic enzyme secretion. ATPase-dependent efflux mechanisms have been suggested as transportation of secreted aspartyl proteinase (SAP) into membrane bound vesicles and upregulation of this transport mechanism might enhance the Sap activity of *C. albicans* (Wu *et al*, 2000). We suggest the significant decrease in germ tube formation,

proteinase and phospholipase activities were the result of the inhibitory effect of cinnamaldehyde on ATPase-dependent efflux mechanisms. Cinnamaldehyde at sub-inhibitory concentrations caused a significant reduction in adhesion to buccal epithelial cells in our study. This suggests cinnamaldehyde could interfere with the adhesion properties of *C. albicans*.

An *in vitro* study of cytotoxicity showed cinnamaldehyde at MIC₉₀ (320 mg/ml) has almost negligible toxicity against H9c2 rat cardiac myoblasts compared to fluconazole at the same concentration (Shreaz *et al.*, 2011a). Moreover, Taguchi *et al.* (2011) reported the therapeutic efficacy of a combination of cassia or cinnamaldehyde and methylcellulose on murine oral candidiasis. These studies suggest the potential therapeutic application of this compound.

In our study, cinnamaldehyde exhibited fungicidal activity and inhibited germ tube formation, adhesion to epithelial cells and hydrolytic enzyme secretion of pathogenic *C. albicans*. The inhibition of those virulence factors by cinnamaldehyde may be beneficial in treatment of candidiasis. Further *in vitro* studies of the antifungal mechanism of cinnamaldehyde are needed.

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