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Eugenol Affects the Germ Tube Formation and Cell Adhesion of *Candida albicans*

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Abstract

Candida albicans is the most common fungal pathogen in humans. Antimicrobial resistance in *C. albicans* is increasingly reported. The antifungal activity of eugenol against clinically relevant fungi including *C. albicans* has been recently described. In this study, the antifungal effects of eugenol on major virulence factors of *C. albicans* were evaluated. *C. albicans* isolates were exposed to sub-MIC of eugenol. The crystal violet-based method was used to quantify the germ tube formation. Extracellular enzymatic activity (proteinase, phospholipase, and lipase) was determined using the agar plate test. The adhesion to buccal epithelial cells was monitored microscopically. Our result showed that eugenol possesses fungicidal activity against *C. albicans*. At sub-inhibitory doses, eugenol significantly suppressed germ tube formation and cell adhesion of *C. albicans* ($p<0.05$). However, the enzymatic activity of yeast cells exposed to eugenol was slightly reduced. Our data indicate the inhibitory effect of eugenol on the growth and the pathogenicity of *C. albicans* in terms of germ tube formation and adhesion.

Keywords: Eugenol, Germ Tube Formation, Hydrolytic Enzymes, Adhesion, *Candida albicans*

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INTRODUCTION

Candida albicans is commonly found as a harmless commensal in the gastrointestinal tract, oral cavity, and genital area. It is responsible for various clinical manifestations in immunocompromised patients varying from mucocutaneous infection to invasive systemic infection.[1] Several virulence factors including adhesion to host tissue, dimorphic transition, and production of extracellular enzymes may contribute to the transformation from harmless commensal to virulent pathogen.[2] Adhesion to the host tissue is a crucial initiation step of colonization and infection. Short hyphal elements called "germ tubes" are important to the adhesion of *Candida* to the host epithelium. Moreover, the organism's hyphal forms contribute to invasion and tissue destruction.[3] Extracellular hydrolytic enzymes play an important role in nutrition acquisition, invasion, tissue injury, and host defense evasion.[4-7] The treatments of candidiasis with polyenes, azole, and echinocandins are satisfactory. However, these antifungal drugs also have many disadvantages regarding their host toxicity, spectrum of activity, and pharmacokinetic properties. Furthermore, infections by multidrug-resistant *C. albicans* have been recently reported.[8] Thus, antibiotics with a new mode of action are urgently needed.[9]

Previous studies showed that eugenol, the major active ingredient of clove oil, has antimicrobial activity against many microorganisms including *Candida* sp. It was found that eugenol was not only able to reduce the growth but also the virulence of *Candida* sp. such as cell surface hydrophobicity and biofilm formation.[10,11] Here, we investigated the effect of eugenol on germ tube formation, extracellular hydrolytic enzyme, and adhesion to epithelial cells of *C. albicans*.

MATERIALS AND METHODS

Strains of Tested Microorganisms

Two reference strains (ATCC 10231 and 90028) and twenty clinical *C.albicans* isolates obtained from blood, urine, genital swab, and body fluids were used. Tested microorganisms were identified by phenotypic analysis including germ tube and chlamydoconidia production, culture on CHROMagar™ *Candida*, growth at 45°C, and carbohydrate assimilation and fermentation test. Before use, yeast cells were grown for 48 h at 37°C on a Sabouraud dextrose agar plate (SDA) (Oxoid®, USA).

MIC and MFC Assay

To determine the MIC of eugenol, the broth microdilution method was performed according to a method of EUCAST (EDef 7.1) with some modifications. Eugenol was purchased from Fluka (Steinheim, Germany). Briefly, eugenol was serially diluted in double-strength RPMI 1640 (Biochrom AG, Germany) containing 4% glucose and 2% DMSO (2 x RPMI-GD) to achieve a range of concentration from 1.95 to 1,000 μg/ml. Yeast suspension (1 x 10^4 CFU/ml) was added with an equal volume of serially diluted eugenol. After incubation at 37°C for 24 hours, yeast growth was determined by using spectrophotometry. The lowest concentration of eugenol inhibiting yeast cells growth was considered the MIC value. MFC was determined by sub-culturing 100 μl from the optically clear well onto SDA. The MFC was considered as the lowest eugenol concentration that kill ≥99.9% of the initial inoculum. All analyses were repeated three times with duplicate determinations.

Time-kill Assay

Time-kill assay was conducted as previously described with some modifications.[12] Yeast cells (5 x 10^4 CFU/ml) of *C. albicans* ATCC 10231 were grown in RPMI-GD in the presence of eugenol at a final concentration of 0, 1/4, 1/2, and 1 x MIC at 37°C. The viability of the yeast cells (CFU/ml) at a different time point was determined by the colony count method. The analysis was independently repeated two times with duplicate determinations.

Inhibitory Effect of Eugenol on Germ Tube Formation

Inhibitory effect of eugenol on germ tube formation was determined by method described elsewhere with modification.[13] Cell suspension (1 x 10^5 CFU/ml) of fresh grown *C. albicans* was prepared and then a 100-μl aliquot was added to the wells of a 96-well, flat-bottom plate containing RPMI-GD (100 μl) in the presence of 10% fetal bovine serum (Gibco BRL, USA) and different

eugenol concentration (0, 0.25, or 0.5 x MIC). A blank RMPI-GD well without yeast cells was included. After incubation for 2 h at 37°C, the medium was discarded and wells were washed once with 100 μl of 70% ethanol. A 200-μl aliquot of 0.25% sodium dodecyl sulfate (SDS) was added to each well to removed yeast cells without a germ tube. Then, the plates were washed thrice with distilled water. Germ tube formation was quantified by using the crystal violet-based technique. To minimize background interference, the absorbance values of 570 nm of the blank were subtracted from the values of the test wells. Three independent experiments with duplicate determinations were performed.

Effect of Eugenol on Hydrolytic Enzyme Activity

The effects of eugenol on activities of hydrolytic enzymes including proteinase, phospholipase, and lipase were determined by using agar containing bovine serum albumin,[14] egg yolk,[15] and Tween-80.[16] Five microliters of 1×10^6 CFU/ml cell suspension were spotted on assay agar containing either 0 (control), 0.25,

or 0.5 x MIC of eugenol. The agar plates were incubated in a humidity chamber at 37°C for 5, 2, and 7 days, respectively. Enzymatic activity (PZ) was determined by the ratio of colony diameter to diameter of colony plus the precipitation or degradation zone. Experiment was performed in triplicate and repeated twice.

Effect of Eugenol on Candidal Adhesion to Buccal Epithelial Cell

Yeast cells (5×10^5 CFU/ml) of *C. albicans* ATCC 10231 were incubated in RPMI-DG with eugenol at concentrations of 0, 0.25, and $0.5 \times$ MIC at 37°C for 1 h. Treated cells were harvested by then centrifugation and washed twice with NSS. Yeast cells were resuspended in NSS and adjusted to yield approximately 1×10^7 CFU/ml. Buccal epithelial cells (BEC) were collected by gently rubbing on the mucosal surface of the cheeks of three healthy volunteers with a sterile swab and pooled in sterile NSS. The BEC was washed three times and adjusted to a concentration of 1×10^5 CFU/ml in NSS. Then, the candidal adhesion to the human buccal epithelial
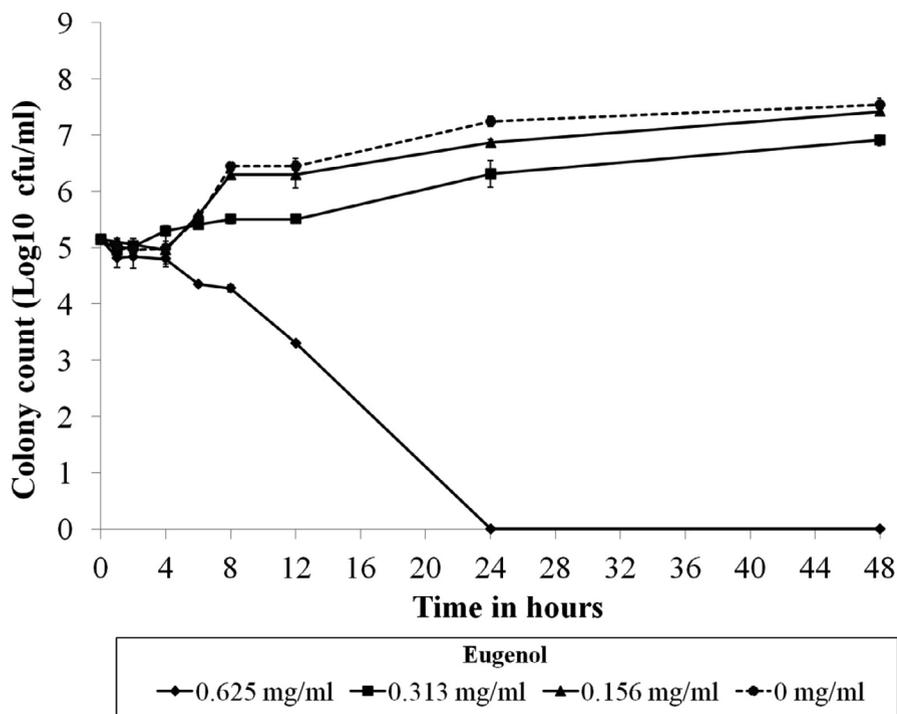

Figure 1. Kinetics of inhibition of *C. albicans* ATCC 10231 by eugenol

cell was performed according to the previously described method.[17] The number of yeast cells attached to 100 BECs was counted using light microscopy at 400x magnification. Experiment was performed in triplicate and repeated thrice.

Statistical Analysis

To evaluate the difference between the control and eugenol treated groups, Wilcoxon Signed Rank Test was used to analyze the effect

Table 1. Germ tube formation of *C. albicans* (n=22) in the presence of eugenol determined by the crystal violet technic measured at OD_{570}

Eugenol (µg/ml)	Germ tube formation (OD_{570})	
	Mean ± SD	Range
0	0.31 ± 0.06	(0.19–0.42)
156	0.09 ± 0.08[a]	(0.01–0.31)
313	0.06 ± 0.05[a]	(0.01–0.20)

[a]$p<0.05$, SD, standard deviation; OD_{570}, optical density at 570 nm.

of eugenol on virulence factors. A *p*-value < 0.05 was considered significant.

RESULTS

MIC, MFC, and Antifungal Activity of Eugenol

The MICs and MFCs of eugenol against twenty-two *C. albicans* strains were equal to 625 µg/ml. Due to the well-known genetic background of *C. albicans* ATCC 10231, this strain was selected for time-kill analysis. The obtained result demonstrated that eugenol at MIC was fungicidal within 24 hours because cell viability was decreased by more than 3 log10 CFU/ml relative to the initial inoculum (Figure 1).

Inhibitory Effect of Eugenol on Germ Tube Formation

The crystal violet-based method was used to evaluate the effect of eugenol on germ tube formation of *C. albicans*. Eugenol cause a significant decrease in OD570 from 0.31 (range: 0.19–0.42) of control to 0.09 (range: 0.01–0.31)
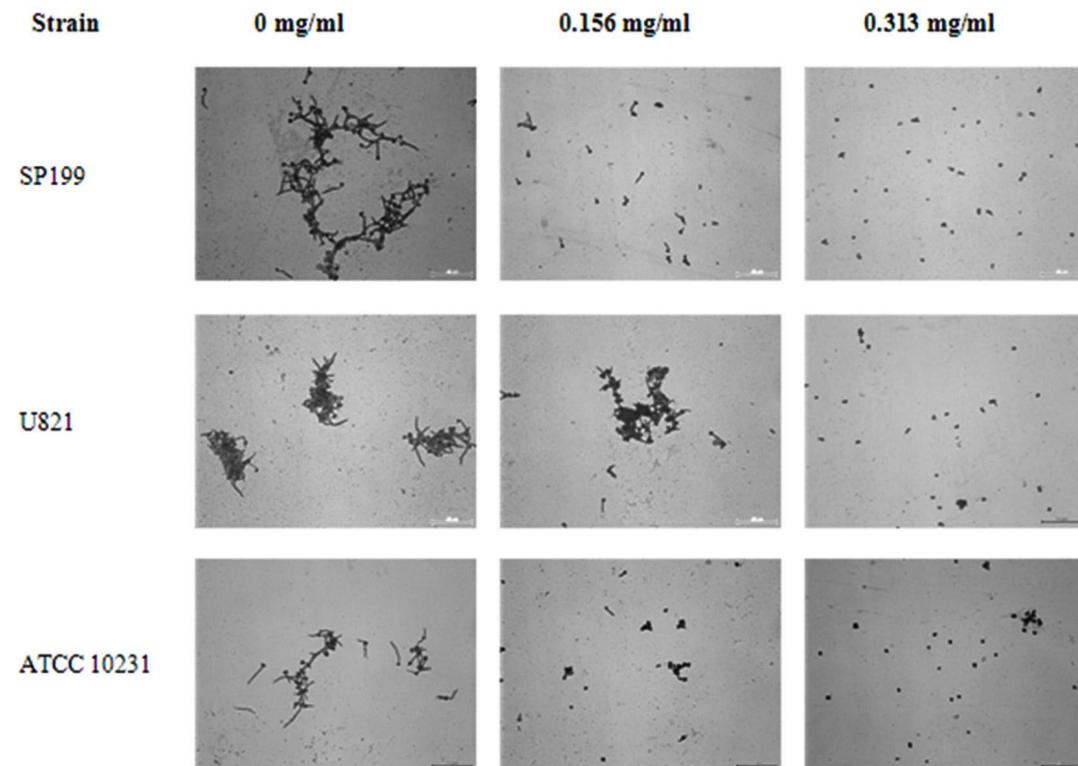

Figure 2. Stained *C. albicans* cell at 100 x magnification exposed to eugenol

and 0.06 (range: 0.01–0.20) at 156 and 313 µg/ml eugenol, respectively ($p < 0.05$). The inhibitory effect of eugenol on germ tube formation was dose-dependent manner (Table 1). The photograph depicting the germ formation of yeast cells treated with various concentrations of eugenol is shown in Figure 2.

Effect of Eugenol on Proteinase, Phospholipase, and Lipase Activities

Hydrolase activities of *C. albicans* exposed to varying concentrations of eugenol were determined using hydrolase-specific testing agar. Twenty-one and nineteen strains including two reference strains exhibiting proteinase and phospholipase activities were used in this experiment, respectively. The Pz for *C. albicans* unexposed to eugenol was 0.68 ± 0.09 for proteinase and 0.71± 0.07 for phospholipase. When the yeast was exposed to 156 and 313 µg/ml eugenol, the activities of these enzymes were slightly but statistically significantly reduced to 0.70 ± 0.08 and 0.70 ± 0.07 for proteinase and 0.73 ± 0.08 and 0.75 ± 0.08 for phospholipase, respectively ($p < 0.05$). In addition, the Pz of lipase activity of twenty-two strains of *C. albicans* not

exposed to eugenol was 0.52 ± 0.09. Lipase activity of the tested strains was not reduced when exposed to eugenol at 0.25 and 0.5 × MIC (0.52 ± 0.09 and 0.53 ± 0.09, respectively, Table 2).

Effect of Eugenol on *C. albicans* Adhesion to the BEC

Compared with the control, eugenol at 0.25 x and 0.5 x MIC inhibited the adhesion activity of *C. albicans* from 180 ± 10.8 yeast cells/100 BEC to 67 ± 5.2 and 36 ± 3.9, respectively ($p < 0.05$). The result showed that the reduction of BEC adhesion of *C. albicans* by eugenol was concentration-dependent (Table 3).

DISCUSSION AND CONCLUSION

Candidiasis has become increasingly significant due to the growing population of immunocompromised patients. Most of the azole drugs are fungistatic. So, treatment provides the chance for acquired resistance to develop in the presence of this drug. New antifungal drugs, especially with a fungicidal mode of action are urgently needed. Eugenol, which is a monohydric phenol compound widely used as a

Table 2. Enzymatic activity of *C. albicans* in the presence of eugenol. Ratio of colony diameter to diameter of the colony plus degradation or precipitation zone was determined and defined as enzyme activity (Pz)

Eugenol (µg/ml)	Proteinase activity (n=21)		Phospholipase activity (n=19)		Lipase activity (n=22)	
	Mean ± SD	Range	Mean ± SD	Range	Mean ± SD	Range
0	0.68 ± 0.09	0.43–0.82	0.71 ± 0.07	0.57–0.90	0.52 ± 0.09	0.42–0.72
156	0.70 ± 0.08[a]	0.48–0.76	0.73 ± 0.08[a]	0.60–1.00	0.52± 0.09	0.42–0.76
313	0.70 ± 0.07[a]	0.51–0.77	0.75 ± 0.08[a]	0.62–1.00	0.53 ± 0.09	0.43–0.76

[a]$p < 0.05$

Table 3. Adhesion of *C. albicans* ATCC 10231 to buccal epithelial cells in the present of eugenol

Eugenol (µg/ml)	Yeast cells/100 BEC	
	Mean ± SD	Range
0	180 ± 10.8	168–188
156	67 ± 5.2[a]	62–72
313	36 ± 3.9[a]	33–40

[a]$p < 0.05$; BEC: buccal epithelial cell; SD: standard deviation.

local antiseptic and anesthetic, has been found to have antimicrobial activity against both planktonic and sessile *Candida* sp.[10,11] As previously reported, our result showed that eugenol has fungicidal activity against *C. albicans*.

Adhesion was considered as the first step in the process leading to persistent colonization, biofilm formation, and establishment of the disease.[19] Eugenol has been reported to reduce cell surface hydrophobicity and adhesion of

C. tropicalis and *C. dubliniensis* to polystyrene and Hep2 cells.[11] In our study, eugenol at sub-inhibitory concentrations significantly suppressed the adhesion of *C. albicans* to buccal epithelial cells. This result emphasized that eugenol could interfere with the adhesion properties of *Candida*. Eugenol may inhibit the synthesis of adhesins or it may cause disruption of adhesion molecules to present on the cell wall and thereby prevent adhesion of the yeast cells.

The secretion of hydrolytic enzymes such as aspartyl proteinases, phospholipases, and lipases during infection is required for the acquisition of nutrients, tissue damage, invasion, and immune system evasion.[4-7] When eugenol was added to the test media, the zones of phospholipase and proteinase activities were slightly reduced. However, no effect of eugenol on lipase activity was observed. A previous study revealed that *Candida* proteinase also plays an auxiliary role of proteinase in cellular adhesion.[20] However, this mechanism can be excluded from our study because eugenol had a limited effect on proteinase activity.

Germ tube formation is a morphological transition process from yeast to hyphal form, leading to tissue invasion, evasion of phagocytosis by macrophage, and biofilm formation.[21-23] *C. albicans* mutants locked in yeast form were avirulent in an animal model.[24-26] In this study, eugenol suppressed the germ tube formation in the presence of serum, which is a hyphae-inducing agent, indicating a significant role of eugenol in hyphal growth inhibition and thereby invasive and biofilm formation capacity.

It has been found that eugenol inhibits ergosterol biosynthesis in *Candida*,[27] also perturbs the activity of amino acid permeases in yeast resulting in cell death and subsequent cytoplasmic leakage.[28] In addition, it has been proposed that eugenol may disturb cellular fluidity and permeability by inserting itself between the fatty acyl chains of the membrane lipid bilayer.[29] By that proposed mechanism, eugenol may suppress yeast-to-hypha transition and alter the cell surface to mask adhesins. Nevertheless, this is speculative and requires further clarification.

Because azole drugs and eugenol have different targets. The latter was found to be still effective against different species of *Candida* sp.

with intrinsic or acquired resistance to fluconazole. Eugenol also exhibited *in vitro* synergy with fluconazole and amphotericin B against *Candida* sp.[30-32] Moreover, the efficacy of eugenol as antifungal prophylaxis and treatment of oral and vaginal candidiasis in an immunosuppressed rat model has been reported.[33] The results obtained in this study demonstrated that eugenol possesses fungicidal activity against *C. albicans* and has inhibitory effects on significant virulence factors of *C. albicans* involved in germ tube formation and adhesion to epithelial cells.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS' CONTRIBUTION

AP designed the study, performed the experiments, analyzed the data, and wrote the manuscript. NM and BN carried out the sampling and culture method. CC and PJ performed the experiments. All authors read and approved the final manuscript for publication.

DATA AVAILABILITY

All datasets generated or analyzed during this study are included in the manuscript.

ETHICS STATEMENT

This study was approved by the Ethical Committee of Thammasat University, Project no. 046/2015.

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