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In vitro antifungal effect of eugenol in combination with fluconazole against *Candida* sp.

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ABSTRACT

Aims: A combination of the antimicrobial drug with the herbal derived antifungal agent was exploited as alternative therapeutic approaches for infectious diseases caused by drug resistant strains. In this study, we determine the antifungal effects of eugenol alone and in combination with fluconazole against *Candida* sp.
Methodology and results: *Candida* strains including fluconazole resistant (*C. parapsilosis* ATCC 22019 and *C. albicans* U821/10) and susceptible strains (*C. tropicalis* U624/10 and *C. glabrata* U71/1) were used in this study. By broth microdilution technique, eugenol exhibited antifungal activity with MIC and MFC against *Candida* sp. tested ranging from 0.5-1 mg/mL. The interaction between eugenol and fluconazole against *Candida* sp. was determined by chequerboard microtiter technic. Eugenol decreased the MIC of fluconazole against *Candida* sp. tested. No antagonism was observed in strains test.
Conclusion, significance and impact of study: From these results, eugenol displayed a promising antifungal effect alone as well as combination with fluconazole against *Candida* sp.

Keywords: Eugenol, fluconazole, *Candida*, drug combination

INTRODUCTION

Candida is a commensal yeast found in the human skin and mucosal membrane surfaces. However, it was found as common opportunistic fungi causing infection of oral, vaginal, and systemic candidiasis. *C. albicans* is the predominant causative organism of almost types of candidiasis, but other emerging *Candida* sp. including *C. glabrata*, *C. krusei*, *C. tropicalis*, and *C. parapsilosis* are now posing serious nosocomial threats to patient populations (Chakrabarti *et al.*, 2009; Xiao *et al.*, 2018). Amphotericin B and fluconazole have been the drug of choice in the treatment of these fungal infections. However, amphotericin B is high nephro- and hepato-toxicity (Groll and Kolve, 2004). Due to its high solubility, low toxicity, and wide tissue distribution, fluconazole is the widely used for systemic candidiasis (Brammer *et al.*, 1990). But fluconazole failures in the treatment of candidiasis have been observed due to intrinsically resistant *Candida* sp. such as *C. krusei* and *C. glabrata* and acquired resistant strains of *C. albicans* (Sobel *et al.*, 2003). New therapeutic strategies are required to overcome these problems. The efficacy of antifungal agents including fluconazole can be improved by using drug combination therapy (Mukherjee *et al.*, 2005). Such therapy has potential advantages over monotherapy in terms of reducing dose-related toxicity and emergence of drug resistance. Eugenol, a major active ingredient of clove oil, have been found to have the antifungal activity against *Candida* sp. with low cytotoxicity (He *et al.*, 2007). In this study, we evaluated the antifungal effect of eugenol in combination with fluconazole against *Candida* sp. *in vitro.*

MATERIALS AND METHODS

Candida sp.

Besides one standard laboratory strain *C. parapsilosis* ATCC 22019 used as a quality control, clinical strain *C. tropicalis* (U624/10), *C. glabrata* (U71/1) and *C. albicans* (U821/10) were used in this study. Clinical strains were obtained from a urine sample in pure culture at $>10^4$ CFU/mL were collected at Microbiology Laboratory Unit, Thammasat University Hospital. The strains were identified by using Brilliance^{TM} Candida Chromogenic agar (Oxoid, Hampshire, UK) and sequencing of the internal transcribed spacer (ITS) of ribosomal DNA. The

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strains were cultured on Sabouraud dextrose agar (SDA) (Oxoid, Hampshire, UK) and incubated at 37 °C for 48 h before use.

Determination of minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MBC) of eugenol and fluconazole

Eugenol (Fluka, Steinhein, Germany) and fluconazole (Sigma-Aldrich, Missouri, USA) were used in this study. Eugenol or fluconazole was diluted in 1% DMSO and used in the assay. The minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) of eugenol and fluconazole against *Candida* sp were determined by broth microdilution technic according to EUCAST (EDef 7.1) (Rodriguez-Tudela *et al.*, 2008). Briefly, yeast suspension was prepared in sterile 0.85% normal saline solution (NSS) and then the concentration was adjusted to 5×10^5 CFU/mL in sterile distilled water. The compound was diluted twofold in double-strength RPMI 1640 (Biochrom AG, Germany) with 4% glucose and 2% DMSO as solvent to achieve a range of concentration from 0.25-8 mg/mL for eugenol and 16-512 µg/mL for fluconazole. An equal volume of yeast suspension (5×10^5 CFU/mL) was added. Drug-free control and cell-free were included as growth and sterility control. The suspensions were incubated at 37 °C for 24 h. Optical density at 630 nm of the suspension was determined using a microplate reader. For the fluconazole, the MIC was calculated based on the density of the growth control and defined as the lowest concentration that results in at least 50% reduction in growth compared with that of the drug-free growth control. MIC of eugenol was defined as the lowest concentration that inhibited visible growth. Sub-culturing the optically clear well was performed to determine the MFC. The experiments were performed in duplicate and repeated three times.

Chequerboard microdilution assay

The interaction of eugenol with fluconazole was evaluated using the chequerboard microdilution assay in 96-well microtiter plates according to methods described elsewhere (Vitale *et al.*, 2005). Briefly, eugenol or fluconazole were serially two-fold diluted in in double-strength RPMI 1640 (Biochrom AG, Germany) with 4% glucose and 2% DMSO to obtain four-times the final concentration to be achieved in the microtiter well. Furthermore, 50 µL of each dilution of eugenol was added to the microtiter well plates in the vertical direction, while 50 µL of each dilution of fluconazole was added in the horizontal direction. One hundred microliters of yeast suspension (2.5×10^5 CFU/mL) was added to each well. Plates were incubated at 37 °C for 24 h. Optical density at 630 nm (OD630) of the suspension was determined using a microplate reader.

To assess the interaction of combinations of drugs the data obtained spectrophotometrically were further analyzed using the fractional inhibitory concentration index (FICI). FICI was defined as the following equation:

$FICI = FIC_E + FIC_F$

Where FIC_E = MIC of eugenol in combination/MIC of eugenol alone

FIC_F = MIC of fluconazole in combination /MIC of fluconazole alone

According to EUCAST (2000) a synergistic effect (SynE) is observed when FICI value $\leq$ 0.5; an additive effect (AddE) when 0.5 < FICI value $\leq$1; an indifferent effect (IndE) when 1 < FICI value < 2 and an antagonistic effect (AntE) when FICI value ≥ 2.

RESULTS

Candida infection is becoming more difficult to treat with antibiotic monotherapy because of an emergence of drug resistant strain. Thus, an effective and safe antifungal agent is required. The present study was conducted to evaluate the efficacy of herbal derived eugenol against *Candida* sp. and analyzes their interaction with fluconazole.

Minimum inhibition concentrations (MICs) and minimum fungicidal concentrations (MFCs) for eugenol and fluconazole against *Candida* sp.

Different *Candida* sp. including *C. tropicalis* U624/10, *C. glabrata* U71/11, *C. parapsilosis* ATCC 22019 and *C. albicans* U821/10 were used in this study. The MIC for fluconazole against *C. tropicalis* U624/10, *C. glabrata* U71/11, *C. parapsilosis* ATCC 22019, *C. albicans* U821/10 were 128, 128, 1 and 0.25 µg/mL, respectively. This result is consistent to the previous study reported that *C. tropicalis* U624/10 and *C. glabrata* U71/11 were considered as resistance strains to fluconazole whereas *C. parapsilosis* ATCC 22019 and *C. albicans* U821/1 were susceptible strains (Wongdech *et al.*, 2018). Azole binds to ferric ion moiety and inhibits the activity of 14 α-demethylase (Erg11), disrupting the ergosterol biosynthetic pathway. Accumulated toxic-intermediate 4α-methyl sterols alter membrane stability, permeability, and the action of membrane-bound enzymes. However, most of the azole drugs are fungistatic and fail to completely eliminate yeast, leading the acquired resistance of *C. albicans* to azoles.

In our study, MIC and MFC for eugenol against *C. tropicalis* U624/10, *C. glabrata* U71/11, and *C. albicans* U821/10 were 1 mg/mL and 0.5 mg/mL for *C. parapsilosis* ATCC 22019 (Table 1) indicating antifungal activity of eugenol against both fluconazole susceptible and resistant *Candida* sp. This result confirmed that eugenol is effective against resistance mechanisms exhibited by *Candida* sp. (Ahmad *et al.*, 2010a; Khan *et al.*, 2012). It has been proposed that eugenol, monohydric phenol with lipophilic nature, may enter between the fatty acyl chains of the membrane lipid bilayer, disturbing its fluidity and permeability (Latifah-Munirah *et al.*, 2015) and also found

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to inhibit an ergosterol biosynthesis in *Candida.* (Ahmad *et al.*, 2010b) Additional, eugenol also perturbed the activity of amino acid permeases in yeast especially Gap1p which plays a role in amino acid sensing in a protein kinase A (PKA)-mediated protein phosphorylation cascade, resulting in cell death and subsequent cytoplasmic leakage (Darvishi *et al.*, 2013). Because of its different target from azole drug, eugenol is seemed to be useful against clinical resistant isolates of *Candida* sp.

Table 1: Minimum inhibition concentration (MIC) and minimum fungicidal concentration (MFC) for eugenol and fluconazole against *Candida* sp.

Microorganism	MIC		MFC
	fluconazole (µg/mL)	eugenol (mg/mL)	eugenol (mg/mL)
C. tropicalis U624/10	128	1	1
C. glabata U71/11	128	1	1
C. parapsilosis ATCC 22019	0.5	0.5	0.5
C. albicans U821/10	0.125	1	1

Fluconazole susceptibility of *Candida* sp. in combination with eugenol

The fluconazole susceptibilities of *Candida* sp. in combination with eugenol were shown in Table 2 and Figure 1. MIC of fluconazole in combination with eugenol was reduced, especially in resistant strains. FICI values for eugenol in combination with fluconazole against all *Candida* sp. tested ranged from 0.6 to 1. It indicated the additive interaction between eugenol and fluconazole. Moreover, no antagonistic interaction was found. This result emphasized a previous study reported that the combination of eugenol with fluconazole reduced the MIC of fluconazole against *Candida* sp. (Ahmad *et al.*, 2010a). Eugenol may increase the susceptibility of *Candida* sp. to the accumulated toxic-intermediate sterols, impair fluconazole export by drug efflux pump or increase the import of azoles through the perturbed membrane. However, our study did not demonstrate the synergistic interaction of eugenol with fluconazole against *Candida* isolates as reported in the previous study (Ahmad *et al.*, 2010a; Khan *et al.*, 2012). In addition to the differences in *Candida* isolates tested, we believed that the inconsistency may be due to the different procedure used in chequerboard microdilution assay. The previous study used the protocol according to CLSI in chequerboard microdilution assay, while we used the protocol suggested by EUCAST. Although, the EUCAST and CLSI protocol were found to produce comparable results for testing the active antifungal drug including fluconazole (Pfaller *et al.*, 2014). The agreement between these methods used for drug interaction study was unknown. Thus, the comparison study of chequerboard microdilution assay using the method according to CLSI and EUCAST should be performed further.

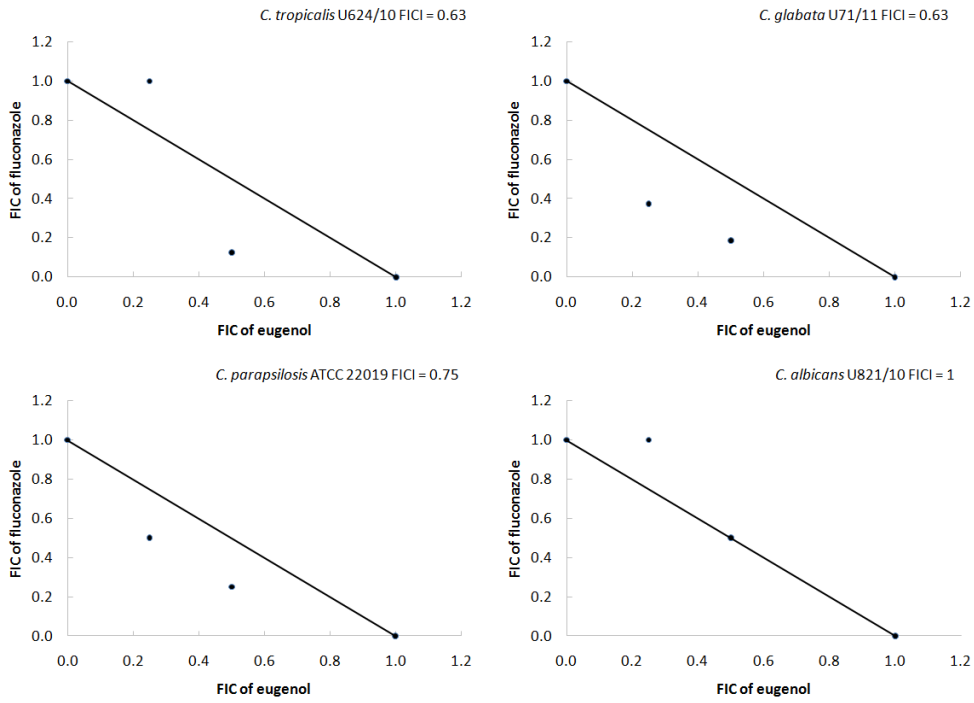

Figure 1: Isobologram of the FIC of eugenol and fluconazole against *Candida* sp. along with FICI values.

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Table 2: Susceptibilities of *Candida* strains used in this study to eugenol in combination with fluconazole.

Microorganism	Agent	MIC_A	MIC_C	FIC	FICI	Interpretation
C. tropicalis U624/10	eugenol (mg/mL)	1	0.5	0.5	0.63	AddE
	fluconazole (µg/mL)	128	16	0.13		
C. glabata U71/11	eugenol (mg/mL)	1	0.5	0.5	0.63	AddE
	fluconazole (µg/mL)	128	16	0.13		
C. parapsilosis ATCC 22019	eugenol (mg/mL)	0.5	0.25	0.5	0.75	AddE
	fluconazole (µg/mL)	1	0.25	0.25		
C. albicans U821/10	eugenol (mg/mL)	1	0.5	0.5	1.00	AddE
	fluconazole (µg/mL)	0.125	0.0625	0.5		

MIC_A, MIC of the agent alone; MIC_C, MIC of the agent in combination; AddE, Additive effect

CONCLUSION

In conclusion, these findings revealed that eugenol had the considerable antifungal activity against different species of *Candida* and also increased the susceptibility of *Candida* sp. to fluconazole. Antimicrobial activity against other common fungal pathogens must be studied in order to evaluate the potential of these compounds for therapeutic applications.

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