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Antifungal potential of eugenyl acetate against clinical isolates of *Candida* species

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ABSTRACT

The study evaluated the efficiency of eugenyl acetate (EA), a phytochemical in clove essential oil, against clinical isolates of *Candida albicans*, *Candida parapsilosis*, *Candida tropicalis*, and *Candida glabrata*. Minimum inhibitory concentrations (MIC) of EA against *Candida* isolates were in the range between 0.1% and 0.4% (v/v). Spot assay further confirmed the susceptibility of *Candida* isolates to the compound upon treatment with respective 1 × MIC. Growth profile measured in time kill study evidence that the compound at 1 × MIC and 1/2 × MIC retarded the growth of *Candida* cells, divulging the fungicidal activity. Light microscopic observation demonstrated that upon treated with EA, rough cell morphology, cell damage, and fragmented patterns were observed in *C. albicans*, *C. parapsilosis*, *C. tropicalis*, and *C. glabrata*. Furthermore, unusual morphological changes of the organism were observed in scanning electron microscopic study. Therefore, it is validated that the compound could cause cell damage resulting in the cell death of *Candida* clinical isolates. Eventually, the compound at sub-MIC (0.0125% v/v) significantly inhibited serum-induced germ tube formation by *C. albicans*. Eugenyl acetate inhibited biofilm forming ability of the organisms as well as reduced the adherence of *Candida* cells to HaCaT keratinocytes cells. In addition, upon treatment with EA, the phagocytic activity of macrophages was increased significantly against *C. albicans* ($P < 0.05$). The results demonstrated the potential of EA as a valuable phytochemical to fight against emerging *Candida* infections.

1. Introduction

Candidiasis caused by the genus Candida, is considered as a most prevalent fungal infection in human [1] and increased frequency of such an infection, especially in immunocompromised patients are being reported worldwide [2]. *Candida albicans* is a well-known dimorphic human pathogenic fungus, recognized as a major causative agent of candidiasis among other *Candida* species. Being a commensal organism in human, under a favorable environmental condition, C. *albicans* causes superficial infections such as vulvovaginal candidiasis [3,4] and oral candidiasis [5,6]. It has been reported that approximately 90% patients with AIDS are being affected with oral candidiasis [7,8], and three fourth of the women population worldwide are susceptible to vaginal candidiasis [6]. In addition, the emerging infection caused by non-*albicans Candida* such as *C. tropicalis*, *Candida parapsilosis*, and *Candida glabrata* has become a threat worldwide [9–11]. *Candida tropicalis* [12] and *C. glabrata* are the most common causative agents of fungemia in cancer patients [13] as well as leads to candidemia. *Candida parapsilosis* is another *Candida* spp. frequently isolated from blood samples and responsible for candidemia [9]. Despite the fact that use of antifungal drugs is considered as a choice for the treatment of *Candida* infections, availability of a limited number of antifungal agents and incidence of resistance development by these organisms towards antifungal drug treatment [9,14–16] necessitates the hunt for an alternative approach to eradicate candidiasis.

Plants are the valuable source of bioactive metabolites possessing various biological activities. Hence, targeting plant-derived

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products could be a suitable substitute to available conventional antifungal therapies [17]. Essential oils derived from plants are one such therapeutic agent exhibits various pharmacological activities including antifungal activity [18–20]. A few examples of essential oil and derived components exhibiting anti-*Candidal* activity includes lemongrass oil [18], essential oil of *Myrtus communis* [19], *Coriandrum sativum* [21], Guatemalan medicinal plants [20], citral [18], carvacrol, and thymol [22]. In addition, the essential oil of *Myrtus communis* [19] exhibited anti-biofilm activity against the *Candida* isolates. Therefore, essential oils and their derived components could be utilized for the treatment of candidiasis.

Essential oil obtained from a medicinal plant, *Syzygium aromaticum* and eugenol, a major phytochemical constituent of the oil have been reported for their growth inhibitory and anti-virulence activities against various *Candida* spp [23]. Eugenyl acetate (EA), another major phytochemical constituent of the essential oil exhibits antibacterial [24], antioxidant [25], and anti-virulence activities [26]. The present work was attempted to evaluate the growth inhibitory activity of EA against clinically isolated *Candida* spp. Furthermore, the effects of the compound on cellular morphogenesis of the fungal isolates were observed. Eventually, anti-virulence potential of the compound against *Candida albicans* was studied.

2. Materials and methods

2.1. Compound preparation

Eugenyl acetate was purchased from Sigma-Aldrich, Indonesia. A stock solution of EA was prepared in ethanol at a final concentration of 100 μl ml^{-1}, before being stored at 4 °C.

2.2. Target strain and culture condition

Following clinical isolates of *Candida* spp. were obtained from Songklanakarind Hospital, Hat Yai, Songkhla and used as target fungi against which the potential of eugenyl acetate was evaluated. The cultures include *C. albicans* NPRCoE 16120 (from sputum), *C. parapsilosis* NPRCoE 16201 (from blood), *C. glabrata* NPRCoE 16401 (from urinary catheter), *C. tropicalis* NPRCoE 16301 (from tissue), *C. tropicalis* NPRCoE 16302, *C. tropicalis* NPRCoE 16303, *C. tropicalis* NPRCoE 16304, and *C. tropicalis* NPRCoE 16305 (from urinary catheter). *Candida albicans* ATCC 90028 was used as a reference fungal strain. The pure culture of *Candida* isolates was maintained in Sabouraud Dextrose Agar (SDA) plate. The cells were cultured overnight in Sabouraud Dextrose broth (SDB) (Hi-Media) at 37 °C.

2.3. Minimum inhibitory concentration assay

The MIC assay was carried out by using the broth microdilution method in a microtiter plate (MTP) [27]. Briefly, EA was two-fold diluted in MTP containing 100 μl SDB to give final concentrations ranging from 0.4% to 0.0031% (v/v). Two microliters of diluted *Candida* cells suspension (an OD_{600} of 0.2) was added to each well. Candida cells with ethanol and without EA were maintained as a control. In addition, miconazole, a standard antifungal agent was included as a positive control. The plates were incubated at 37 °C for 24 h and the MIC was determined.

2.4. Spot assay

The assay was carried out by the previously described method [28]. Briefly, overnight grown *Candida* cells were adjusted to 0.8 at OD_{600} and serial ten-fold diluted in sterile SDB. Five microliters of each diluted culture were spotted on Sabouraud Dextrose (SD) top agar containing EA at 1 × MIC. The control without the compound and in the presence of respective solvent was maintained. The plate was incubated for 24 h at 37 °C and observed for the growth inhibition.

2.5. Growth profile study

Two percentage of each *Candida* spp. (An OD_{600} of 0.2) was incubated with 100 μl SDB broth containing respective 1 × MIC and 1/2 × MIC EA in 96-well MTP. The yeast cells with ethanol and without EA were maintained as a control. The plate was incubated at 37 °C and growth OD was monitored at different time intervals up to 24 h at 600 nm [29].

2.6. Light microscopic study

To determine the possible effects of EA on tested *Candida* isolates, the cells were exposed to EA and visualized under a light microscope. Briefly, *Candida* cells were cultivated in SDB, overnight at 37 °C. The cells were pelleted by centrifugation at 3000 rpm for 5 min and the pellet was redissolved in sterile phosphate buffered saline (PBS) (pH 7.4) to attain an OD_{600} of 0.8. Two hundred and fifty microliters of the cell suspension of *Candida* spp. were separately added to each well of 24-well MTP containing 750 μl PBS supplemented with respective MIC of EA. The plates were incubated for 2 h, 4 h, and 6 h at 37 °C. After incubation, 10 μl of EA treated and untreated cells were smeared on clean glass slides and stained with 0.4% crystal violet (CV) solution (0.4 g in 100 ml sterile water). After 10 min of incubation, the slides were washed gently with sterile water to remove excess CV and air dried. The stained cells were viewed under a light microscope at ×100 magnification and images were captured (Olympus BX51 microscope attached to the Olympus DP71 camera).

2.7. Scanning electron microscopic study

Candida albicans ATCC 90028 was treated with EA at 1/2 × MIC as mentioned above. After incubation, cells were pelleted by centrifugation at 3000 rpm for 5 min, dissolved in 20 μl PBS and gently smeared on 1 × 1 cm slide. The cells were prefixed with 3% glutaraldehyde in 0.1 M PBS (pH-7.4) for 2 h and dehydrated with graded series of ice cold alcohol (30–100%). The dehydrated cells were dried and then coated with gold particle. The coated samples were examined using scanning electron microscope (FEI Quanta 400 FEG) at 20 kV to visualize the morphological changes.

2.8. Germ tube assay

The assay was performed by previously described method [30,31]. Briefly, overnight grown *C. albicans* culture was centrifuged at 3000 rpm for 5 min to pelletize the cells. The cells were washed two times with sterile water and adjusted using the medium containing 1% yeast extract, 2% tryptone, and 2% glucose to an OD_{600} of 1. Fifty microliters of the cells were added in the wells of 96-well MTP. Twenty microliters of the same medium containing EA at two different concentrations (0.0125% and 0.00625%) supplemented with 30 μl (30%) fetal bovine serum (FBS) was added. Wells with *C. albicans* and serum along with respective solvent was maintained as a control. The plate was incubated for 6 h at 37 °C. After the incubation, a total of 300 yeast cells were counted from each sample under a light microscope and percentage germ tube formation was calculated. Counting of yeast and germ tube was carried out by following the criteria [32]. Clumped as well as yeast cells with pseudohyphae were excluded, mother cells with an