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Effects of patchouli and cinnamon essential oils on biofilm and hyphae formation by *Candida* species

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ABSTRACT

The prevalence and fatality rates with biofilm-associated candidal infections have remained a challenge to the medical fraternity despite major advances in the field of antifungal therapy. Traditionally, essential oils (EOs) from the aromatic plants have been found to be excellent therapeutic agents to treat fungal ailments. The present study explores the antivirulent and antibiofilm effects of under explored leaf EOs of Indian patchouli EO extracted from *Pogostemon heyneanus* (PH), Indian cassia from *Cinnamomum tamala* (CT) and camphor EO from *C. camphora* (CC) against *Candida* species. The EOs were investigated for its efficacy to disrupt the young and preformed *Candida* spp. biofilms and to inhibit the yeast to hyphal transition, a hallmark virulent trait of *C. albicans*. The ability of these EOs to inhibit metabolically active cells was assessed through XTT assay. Of these three EOs, CT EO showed enhanced biofilm inhibition than others and hence it was further selected to study its biomass inhibition potential and exopolysaccharide layer disruption ability. The CT EO reduced the biomass of the preformed biofilms of all three *Candida* strains, which was supported by confocal microscopy. It also disrupted the exopolysaccharide layer of the *Candida* strains as shown by scanning electron microscopy. The present findings validate the effectiveness of EOs against the virulence of *Candida* spp. and emphasize the pharmaceutical potential of several native but yet unexplored wild aromatic plants in the prospect of therapeutic application.

1. Introduction

Candida spp. are opportunistic fungal pathogens [1], which colonize the vaginal and oral epithelial regions [2] and cause superficial as well as invasive infections [3]. They thrive in a closely packed dense community of cells encased in a self-synthesized extracellular matrix termed "biofilms" which are notorious for forming on indwelling medical devices and lay the patients at an increased risk of infection [4,5]. Antibiotic therapy for device-associated biofilm infections often fails due to the formation of thick exopolysaccharide (EPS) layers by the biofilm cells that prevent the entry of antimicrobials from reaching the cell and thereby renders the pathogen resistant to most of the present day drugs [6]. Therefore, the only viable option for treating device mediated infections is the removal of the biofilm adhered indwelling device by surgical intervention followed by antimicrobial therapy [7,8]. In addition to biofilms, the morphogenetic shift from yeast to hyphal form in dimorphic fungi is considered to be another most unique virulent trait that aids in the invasion, development and maintenance of the architecture of the biofilms on host tissues [9,10]. As *Candida* biofilms are intrinsically resistant to conventional antifungal agents, alternative therapeutics like plant-based natural products have been evaluated for their antibiofilm activity [11,12]. Essential oils (EOs) are complex mixture of volatile compounds extracted from medicinal and aromatic plants that are used in the treatment of several diseases in folklore medicine [13]. Though EOs from several plants have been demonstrated to exhibit a plethora pharmacological activities [14–16], their antibiofilm properties have just recently gained

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importance [17–20]. Though the antivirulent property of the major compound linalool present in some EOs have been reported [21] the antivirulent potential of EOs from aromatic plants like *Pogostemon* spp. and wild *Cinnamomum* spp. are still unexplored. We have recently reported the antibiofilm and antivirulent effects of the leaf EOs of *Pogostemon heyneanus* (PH) (Indian patchouli) and *Cinnamomum tamala* (CT) (Indian cassia) against *Streptococcus pyogenes* and the *Pseudomonas aeruginosa* respectively [17,22]. Since targeting hyphal development and biofilms is considered to be a more effective and tangible measure to alleviate candidal pathogenesis [23], the present study was aimed to investigate the antibiofilm and antivirulent efficacy of underexplored EOs of PH, CT and *Cinnamomum camphora* (CC) against the biofilms of *Candida albicans* and non-albicans strains.

2. Materials and methods

2.1. Fungal strains

The test organism's viz. *C. albicans* (ATCC-90028), *C. glabrata* (MTCC 6507) and *C. tropicalis* (MTCC 310) were maintained in Potato Dextrose Agar (HiMedia, India) plates [8]. These fungal strains were grown overnight in Yeast Peptone Dextrose (YPD) broth at 37 °C. After overnight incubation, yeasts cells were harvested in fresh YPD broth to attain a late exponential growth phase of optical density 0.38 at 620 nm (10^7 cells/mL) for the biofilm assay. Most of the assays were performed according to our previously established protocols for *C. albicans* [8].

2.2. Source of EOs

The leaves of PH, CT and CC were collected from Courtallum hills, Tamil Nadu; Almora, Uttarakhand and Botanical garden of Madras Christian College, Tamil Nadu, respectively. Herbarium voucher specimens (R Murugan 33; KC Sekar 66; R Murugan 90) were prepared and deposited at the herbarium of SASTRA University, Thanjavur. The leaf samples were shade dried and subjected to hydro-distillation using Clevenger apparatus. After distillation, the EOs were isolated and dried over a pinch of anhydrous sodium sulphate and stored under refrigeration at 4 °C. The chemical constituents of these EOs have already been studied and published in our earlier reports [22,24,25].

2.3. Determination of Minimum Inhibitory Concentration (MIC)

The Minimal Inhibitory Concentration (MIC) of EOs against *Candida* spp. was performed in 96-well microtiter plates (NEST biotechnology, Korea) as per Clinical and Laboratory Standards Institute guidelines [26]. *Candida* cells were added in YPD broth supplemented with varying concentrations of EOs (0.1, 0.2, 0.4, 0.6, 0.8, 1.0, 5.0, 10.0 v/v %) which were dissolved in 1% DMSO and incubated at 37 °C for 24 h. Flucnazole, a standard antifungal agent was used as positive control. YPD broth with DMSO alone was used as a vehicle control and YPD broth alone was used as a sterility control. Following incubation, microtiter plates were read spectrophotometrically at 620 nm. The MIC was recorded as the lowest concentration that produced complete suppression of visible growth [27].

2.4. Growth curve assay

The YPD broth containing 1% overnight culture of three *Candida* spp. was supplemented with biofilm inhibitory concentration (BIC) of three EOs and the flask was incubated for 24 h at 37 °C. The flask containing overnight culture without EO served as control. Optical density was read spectrophotometrically at every one-hour interval up to 24 h [8].

2.5. Antibiofilm activity of EOs

2.5.1. Biofilm inhibition efficacy of EOs

The effect of the three EOs to inhibit the biofilms of the three *Candida* spp. was tested in 24-well microtiter plates. The sub-MIC concentration of the EO was added in YPD broth containing the fungal suspension at 10^7 cells/mL and incubated at 37 °C for 24 h. After incubation, the plates were stained using 0.4% crystal violet [8].

2.5.2. Examination of preformed biofilm disruption

The biofilms were formed on 24-well titer plate as mentioned above. To the preformed biofilm, the BIC of EOs were added and incubated further for 24 h at 37 °C. After overnight incubation, the spent media was discarded, and the wells were washed, air dried and stained with 0.4% crystal violet and the absorbance was quantified spectrophotometrically at 595 nm [8]. The percentage inhibition was calculated as follows.

$$\text{Percentage Inhibition} = \left[\frac{\text{Control OD at } 595nm - \text{Test OD at } 595nm}{\text{Control OD at } 595nm}\right] \times 100$$

2.5.3. Determining the cell viability of preformed biofilms

The inhibition of metabolically active cells of *Candida* spp. by three EOs was assessed by XTT [2, 3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide] reduction assay [8]. Fresh broth (900 μl), 90 μL of XTT salt solution (0.5 mg/mL) and 10 μL menadione solution (1 mM) were added to the preformed biofilms. Finally, EOs were added at their respective BIC concentration and the plates were incubated at 37 °C for 5 h under dark condition. The conversion of XTT tetrazolium salt to XTT formazan resulted in a colorimetric change, which was read spectrophotometrically at 490 nm.

2.6. Microscopic techniques

2.6.1. Visualization of matured biofilm inhibition by light microscopy

The biofilms were allowed to form on 1cm × 1 cm glass slides, which were placed inside a 24-well microtitre plate. The BIC of EO was added to the preformed biofilms and the plates were incubated for 24 h at 37 °C. After 24 h incubation, the planktonic cells were removed and the biofilm formed on the glass slides were stained using crystal violet dye for 5 min. It was then gently washed with de-ionized water and allowed to air dry for 5 min. Then, the slides were viewed under a light microscope at a magnification of 40× (Nikon Eclipse Ti 100, Japan) [8].

2.6.2. Inhibition of yeast to hyphal shift in C. albicans

The hyphal growth of *C. albicans* was induced by supplementing YPD broth with 10% fetal bovine serum (FBS). The biofilm was formed as mentioned above and treated with BIC of the respective EOs. The ability of EOs to inhibit the transition from yeast to hyphal form was observed by light microscopy [8]. The cell suspension of *C. albicans* were grown on spider medium containing 10% FBS, supplemented with and without the BIC of the respective EOs. The plates were incubated at 37 °C for 36 h. The morphology of *C. albicans* colony formed on the spider agar medium was visualized using a gel documentation system (Bio-Rad Laboratories, XR+, USA) and the images were captured using Image Lab software [28].

2.6.3. Confocal microscopy

Confocal microscopy was performed to visualize the ability of the EOs to disassemble preformed/matured biofilms for all three strains of *Candida* spp. The EO of PH was targeted against *C. albicans*

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