Archives of Oral Biology 87 (2018) 35–42

Contents lists available at ScienceDirect

Archives of Oral Biology

journal homepage: www.elsevier.com/locate/archoralbio

Effects of *Rhodomyrtus tomentosa* extract on virulence factors of *Candida albicans* and human neutrophil function

Jutharat Hmoteh[a,b], Khadar Syed Musthafa[b], Supayang Piyawan Voravuthikunchai[a,b,*]

[a] Department of Microbiology, Faculty of Science, Prince of Songkla University, Hat Yai, Songkhla, 90112, Thailand
[b] Excellence Research Laboratory on Natural products, Faculty of Science and Natural Product Research Center of Excellence, Prince of Songkla University, Hat Yai, Songkhla, 90112, Thailand

ARTICLE INFO

Keywords:
Rhodomyrtus tomentosa
Candida albicans
Virulence factors
Neutrophils
Immunostimulatory activity

ABSTRACT

Objective: *Candida albicans* has become a major problem of oral candidiasis due to increase in antibiotic resistance. *Rhodomyrtus tomentosa*, a medicinal plant possessing several phytochemical constituents, has been considered as a potential source of antimicrobial and immunomodulatory agents. The aim of this study was to investigate anti-virulence and immunostimulatory activity of *R. tomentosa* ethanolic leaf extract against *C. albicans*.

Methods: The effects of the extract on *C. albicans* were assessed on germ tube production, adherence of the organisms to surfaces, and biofilm. In addition, the effects of the extract on phagocytosis and killing activity of neutrophils against the pathogen were investigated.

Results: Suppression of germ tube production following 30 min exposure to the extract at 256 μg/mL was significantly increased in comparison with that of the unexposed cells ($p < 0.05$). The pathogens demonstrated a significant reduction in adherence ability to surfaces in a dose dependent manner, compared with the control ($p < 0.05$). At 48 h, the extract at 512–1024 μg/mL significantly reduced biofilm forming ability of the organisms up to 42.31–64.58% ($p < 0.05$). Compared with the control group, a significant inhibition of mature biofilm after treated with the extract at 256 μg/mL was observed ($p < 0.05$). The extract at 50 μg/mL significantly enhanced phagocytosis and killing activity of neutrophils against the organism, compared with the control ($p < 0.05$).

Conclusions: The findings suggest that *R. tomentosa* extract displayed a dual mode of action, inhibiting virulence factors of *C. albicans* and enhancing neutrophil functions. Further pharmaceutical development of the extract might be useful as an alternative therapeutic agent against oral candidiasis.

1. Introduction

Oropharyngeal candidiasis caused by pathogenic member of *Candida* species is frequently found in immunocompromised patients. *Candida albicans* is the most predominantly isolated opportunistic pathogen from oral cavities causing candidiasis (Sun et al., 2016). The pathogen has many virulence factors involved in its pathogenesis. The main virulence attributes of the organism include conversion of the yeast form to hyphal form (Phan, Belanger, & Filler, 2000), adhesion (Tomicic & Raspor, 2017), and the formation of a biofilm (Ramage, Vandewalle, Wickes, & Lopez-Ribot, 2001). Fluconazole or other azoles is considered to be the standard treatment of the infections (Pappas et al., 2016). However, resistance of the organisms to antifungal drug is an important public health problem. An alternative approach for the elimination of *C. albicans* infections is urgently required.

Currently, new targets for antifungal agents associated with virulence factors of the pathogen. Plant materials used in traditional medicines are a valuable source of antimicrobial agents (Höfling, Mardegan, Anibal, Furletti, & Foglio, 2011). In addition, several medicinal plants have been shown to enhance immunomodulatory activity through immune cells to eliminate pathogens from host (Búfalo, Bordon-Graciani, Conti, Golim, & Sforcin, 2014). Neutrophils are major phagocytic cells of innate immune system which play important role to destroy invading organisms by phagocytic and killing activity. Myeloperoxidase and NADPH-oxidase, highly potent oxidative enzymes, have been reported in neutrophils. Mice without both enzymes exhibited increase in susceptibility to candidiasis (Aratani et al., 2002).

Rhodomyrtus tomentosa (Aiton) Hassk. is a traditional alternative

* Corresponding author at: Department of Microbiology and Excellence Research Laboratory on Natural Products, Faculty of Science and Natural Product Research Center of Excellence, Prince of Songkla University, Hat Yai, Songkhla, 90112, Thailand.
E-mail address: supayang.v@psu.ac.th (S.P. Voravuthikunchai).

https://doi.org/10.1016/j.archoralbio.2017.11.007
Received 27 April 2017; Received in revised form 31 October 2017; Accepted 16 November 2017

medicine belonging to the family Myrtaceae. *R. tomentosa* extract and its principle compound, rhodomyrtone, possessed anti-staphylococcal biofilm (Saising, Götz, Dube, Ziebandt, & Voravuthikunchai, 2015), antifungal activity against *C. albicans* (Limsuwan et al., 2014), antioxidant (Lavanya, Voravuthikunchai, & Towatana, 2012), and immunomodulatory activities (Hmoteh, Musthafa, Pomwised, & Voravuthikunchai, 2016; Srisuwan, Tongtawe, Srimanote, & Voravuthikunchai, 2014).

In the present study, an attempt was undertaken to investigate the potential of *Rhodomyrtus tomentosa* extract on virulence factors including germ tube formation, adhesion, biofilm formation, and mature biofilm of *C. albicans*. Furthermore, the effect of the extracts on phagocytosis and killing activity of human neutrophils against the pathogen was evaluated.

2. Materials and methods

2.1. Fungal isolates and culture conditions

Candida albicans NPRCoE 160122, NPRCoE 160123, and NPRCoE 160124 were obtained from Excellence Research Laboratory on Natural Products, Faculty of Science and Natural Product Research Center of Excellence Faculty of Science, Prince of Songkla University, Hat Yai, Songkhla, Thailand. *C. albicans* ATCC 90028 and *C. albicans* NCPF 3153 were used as reference strains. Pure culture of all the isolates was maintained in Sabouraud dextrose agar (SDA) (Difco, Detroit, USA). For experimental studies, the fungal isolates were cultured in Sabouraud dextrose broth (SDB) (Difco, Detroit, USA) at 37 °C overnight in a rotary shaker until the cells reach stationary phase. The stationary phase cells were harvested by centrifugation at 2,000 × g for 7 min, washed twice with normal saline solution (NSS), and resuspended in Roswell Park Memorial Institute 1640 medium (RPMI 1640) (Sigma, Missouri, USA). An aliquot was picked and counted using a haemocytometer. The resuspended cells were used for further studies.

2.2. Preparation of Rhodomyrtus tomentosa extract

Fresh leaves of *R. tomentosa* were collected in April of 2013. The sample was dried and reduced to a powder. The dried leaf powder was extracted with 95% ethanol for 7 days. The solvent layer was collected and evaporated to dryness in a rotary evaporator to obtain the crude leaf extract. The stock solution was prepared by dissolving 100 mg of crude extract in one microlitre of dimethyl sulfoxide (DMSO)(Merck, Darmstadt, Germany) and stored at 4 °C until used.

2.3. Effects of R. tomentosa extract on germ tube formation of C. albicans

Yeast cells (1×10^6cells/mL) were pre-incubated along with various concentrations of the extract (0–256 µg/mL) in RPMI 1640 medium. Yeast cells in the same medium along with 1%dimethyl sulfoxide (DMSO) were used as a control. The plate was gently mixed and incubated at 37 °C for 30 min. After incubation, 200 µl of 10% fetal bovine serum was added to the cells to develop a germ tube at 37 °C for 4 h. Amphotericin B was used as positive control. Germ tube formation was quantified under low magnification of light microscope.

2.4. Effects of R. tomentosa extract on adhesion of C. albicans to polystyrene surface

The effect of the extract on adherence of *C. albicans* to 96-well plate was evaluated using resazurin assay (Repp, Menor, & Pettit, 2007). One hundred microlitres of the cell suspensions (1×10^7cells/mL) were cultured with 100 µl of various concentrations of the extract (0–1024 µg/mL). The cell suspension with 1% DMSO was used as a control. The plate was then incubated at 37 °C for 0, 2, and 4 h. After incubation, the extract and free floating cells were removed and adherent cells in the wells were gently washed twice with NSS. The adherent cells were exposed to resazurin solution (Thermo Fisher Scientific Inc, Massachusetts, USA) and incubated at 37 °C for 1 h. Following incubation, the absorbance of the solution was measured at 570 nm and 600 nm by spectrophotometer (Bio-Tek Instruments, Inc., Vermont, USA). The adhesion assay was performed in triplicate, percentage reduction of resazurin as a measure of cell adhesion was determined according to Pettit et al. (2005).

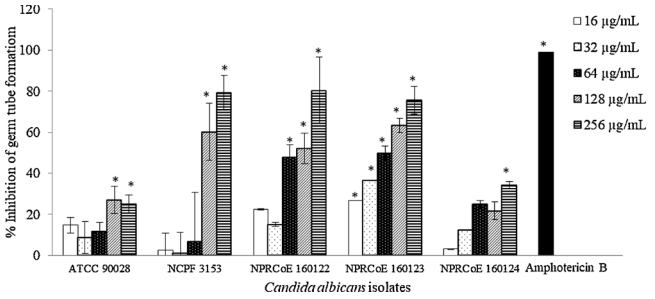

Fig. 1. Inhibitory activity of *Rhodomyrtus tomentosa* extract at 16–256 µg/mL on germ tube formation in *Candida albicans*. Amphotericin B was included as positive control. Values are shown as means of triplicate ± standard error of the mean from two independent experiments. Statistically significant values were defined as * $p < 0.05$.

2.5. Effects of R. tomentosa on biofilm formation of C. albicans

Biofilm assay was performed in 96-well flat bottom microtiter plate (Repp et al., 2007). Briefly, 100 µl of the cell suspensions (1×10^7cells/mL) were cultured with 100 µl of various concentrations of the extract (0–1024 µg/mL) incubated at 37 °C for 24 and 48 h. DMSO was used as a control. After incubation, the plate was washed with NSS to remove non-adherent cells and biofilm in the microtiter plate was quantified by resazurin assay. Briefly, 20 µl of freshly prepared 1% resazurin solution was added to each well and incubated at 37 °C for 1 h. Following incubation, absorbance was measured at 570 nm and 600 nm in spectrophotometer. The experiments were done in triplicate.

2.6. Effects of R. tomentosa on matured C. albicans biofilm

To investigate the effect of the extract on established biofilm, initially, the yeast cells were allowed to develop biofilm on 96-well flat bottom and incubated at 37 °C for 48 h (Repp et al., 2007). Following incubation, the plate was washed two times with NSS and 200 µl of fresh RPMI 1640 medium containing various concentrations of the extract (0–1024 µg/mL) were added into each wells. The medium with 1% DMSO was kept as a control. The plate was further incubated for 24 h at 37 °C. After incubation, the plate was washed with NSS to remove non-adherent cells. The biofilm in the test plate was exposed to resazurin solution and incubated at 37 °C for 1 h. Following incubation, the absorbance of the solution was measured at 570 nm and 600 nm by spectrophotometer. The assay was performed in triplicate.

2.7. Scanning electron microscopy (SEM)

The morphological changes in germ tube formation, biofilm formation, and mature biofilm of the fungal cells after treatment with the extract were investigated by scanning electron microscopy. Briefly, changes in both extract treated (256 µg/mL) and untreated yeast cells form the germ tube inducing serum at 4 h, the biofilm architecture of yeast cells after treatment with the extracts in the absence and presence of the extract (512 and 1024 µg/mL) on glass slide immersed in the 24-well flat bottom containing RPMI 1640 medium. In order to assess the effect of the extract on matured biofilm, yeast cells were initially allowed to form biofilm on glass slide immersed in 24-well flat bottom containing RPMI 1640 medium for 48 h and afterwards this condition