Original Article

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Inhibitory and Anti-Biofilm Effects of *Orthoshipon aristatus* Against *Candida albicans*

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ABSTRACT

There are increasing number of reports on *Candida albicans* developing resistance to available anti-fungal drugs. Thus, there is an urgent need to discover new agents for treatment of candidiasis. The alcoholic extracts of *Orthoshipon aristatus* have been shown to exhibit antifungal activity against *C. albicans* by using the agar diffusion and broth microdilution methods. However, the underlying mechanisms of anti-*C. albicans* effect of *O. aristatus* have not been well understood. This study was aimed to evaluate the cytotoxic and anti-biofilm effects of the n-hexane and ethanol extracts of purple and white varieties of *O. aristatus* leaves and branches against *C. albicans*. The effect of n-hexane and ethanol extract against *C. albicans* growth was carried out by crystal violet viability assay. IC_{50} values of the most active extract, and nystatin and fluconazole as positive controls were also determined by the crystal violet assay. Evaluation of the anti-biofilm effect was performed by treating *C. albicans* with the extracts at adhesion, development, and biofilm maturation stages using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The n-hexane extract of the purple variety of *O. aristatus* leaves demonstrated the strongest cytotoxic activity against *C. albicans* amongst the tested extracts, with an IC_{50} value of 0.67 µg/mL. The extract also showed strong anti-biofilm effect as fluconazole, with pronounced inhibition at the adhesion stage and less activity at the biofilm development and maturation stages. These results suggested that the n-hexane extract of the purple variety of *O. aristatus* leaves could be explored for discovery and development of anti-*C. albicans* agent.

Keywords: *Orthoshipon aristatus; anti-Candida albicans; cytotoxic; anti-biofilm*

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INTRODUCTION

Candida albicans is an oval and unicellular commensal microorganism found in the skin, vaginal and respiratory mucosa. However, it can turn into a pathogen and cause superficial and systemic candidiasis mainly caused by immune suppression (Kadosh & Lopez, 2013). Candidias can usually be suppressed by fluconazole, however in recent years it has been reported that the incidence of *C. albicans* resistance against azole and nystatin has been increasing (Oxman et al., 2010; Mohamadi et al., 2014; Sandai et al., 2016). The presence of biofilms and wide use of azole drugs have been identified as one of the resistance factor (Lortholary et al., 2010). Thus, the emergence of *C. albicans* resistance is the major driving factor for development of novel anti-*Candida albicans* drugs.

Numerous reports showed that natural products such as herbal medicine or its derivatives possess potential antiviral, antimicrobial, or anti-fungal activity (Atanasov et al., 2015). Manuka honey has been reported not only to exhibit antibacterial activity but also antiviral activity (Watanabe et al., 2014). Epigallocatechin gallate was also demonstrated to exhibit the Zika virus growth *in vitro* (Raekiansyah et al., 2018). Furthermore, Rooibos tea showed activity against Influenza virus type A and B (Rahmasari et al., 2017). Consequently, the anti-fungal activities of herbal medicines, and their constituents are of great research interest.

Orthosiphon aristatus is a plant distributed across South East Asia and South Asia. It is also known as kumis kucing in Indonesia, misai kucing in Malaysia, and java tea in United States. It is traditionally used for the treatment of kidney stone bladder, vaginitis, urinary infection, and rheumatoid (Mukesh et al., 2015). There are two varieties of *O. aristatus* grown in Indonesia, namely, white and purple flower. To note, the purple variety is known to contain more bioactive compounds compared to the white variety (Ashraf et al., 2015). In general, *O. aristatus* has been reported to contain the

glycoside orthoshipon, terpenoids, and flavonoids such as eupatorin, sinensetin, salvigenin, ladanein, vomifoliol, 5-hydroxy-6,7,3′,4′- tetramethoxyflavone, 6-hydroxy-5,7,4′-trimethoxyflavone, 7,3′,4′-tri-O-methylluteolin, tetramethylscutellarein and scutellarein tetramethylether (Lyckender & Malterud, 1996; Tezuka et al., 2000). A previous study used the agar diffusion method to demonstrate the anti-*C. albicans* activity of ethanol extract of *O. aristatus* (Romula et al., 2018). However, the effect of direct contact between various extracts of *O. aristatus* and *C. albicans* cells as well as the analysis of the possible mode of action, have not been reported. In the present study, we investigated the anti-*C. albicans* activity of n-hexane and ethanol extracts of both white and purple varieties of *O. aristatus* by evaluating their cytotoxic and anti-biofilms effects. We found that the n-hexane extract of leaves of the purple *O. aristatus* potently suppressed the viability of *C. albicans* and interfered with the adhesion stage in biofilm formation.

MATERIALS AND METHODS

Cells and Samples

Candida albicans ATCC 10231 were grown in a 90 mm-petri dish containing potato dextrose agar (Wako Pure Chemical Industries, Osaka, Japan) and maintained in an incubator at 28ºC. For experimental purposes, *C. albicans* from the petri dish were further transferred into slant potato dextrose agar and incubated at 28ºC for 24 h. Nystatin (Metiska Farma, Jakarta, Indonesia) and fluconazole (Novell Pharmaceutical Laboratories, Jakarta, Indonesia) were dissolved in ultrapure water. Dried of 3 months old *O. aristatus* white and purple varieties were purchased from Balai Penelitian Obat dan Rempah Indonesia on August, 2019 (Bogor, Indonesia) and identified in Lembaga Ilmu Pengetahuan Indonesia (Indonesian Institute of Science, Bogor Indonesia).

Extract Preparation

One gram each of dried branches and leaves of white and purple varieties of *O. aristatus* was extracted with 100 mL of n-hexane (Wako Pure Chemical Industries, Osaka, Japan) and shaken at 37°C for 3 h. After Whatman paper filtration, the filtrate was vacuum evaporated to obtain the n-hexane extract and the residue was further extracted by 96% ethanol (Wako Pure Chemical Industries, Osaka, Japan) and shaken at 37°C for 3 h. After centrifugation at 3000 rpm for 15 min (Tomy Mx-305), the extract was filtrated by Whatman paper and vacuum evaporated to obtain the ethanol extract. The dried extracts were dissolved in DMSO (0.2g/mL) and the final DMSO concentration in each reaction mixture was 1%.

Crystal Violet Viability Assay

The crystal violet viability assay was carried out using a 96 well-culture plate coated with 50% fetal bovine serum (Sigma Aldrich, St. Louis, America). A volume of 100 µL/well of tested extract diluted in sabouraud dextrose broth (SDB) (Wako Pure Chemical Industries, Osaka, Japan) was added into 96 well-culture plates followed by the addition of 100 µL/well of *C. albicans* (3×10^4 cells/well) in SDB. The mixture was then incubated at 28°C for 48 h. The supernatant was then aspirated, and the cells were fixed with 70% ethanol and stained with 0.5% crystal violet. Cells were dried and diluted by 96% ethanol. The absorbance was measured at 460 nm using a microplate reader (VersaMax Molecular Devices, California, USA).

The selected extract of *O. aristatus* which shown highest activity in inhibiting *C. albicans* were subjected to a serial dilution to obtain solutions of 2, 1, 0.5, 0.25, 0.125, 0.062, 0.031 and 0.016 mg/mL. The percentage inhibition was calculated using Microsoft Excel software, and the 50% inhibition concentration (IC_{50}) was determined by linear regression analysis.

Biofilm Susceptibility Assay

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was carried out to evaluate the efficacy of *O. aristatus* extracts in interrupting biofilm formation using 96 well-flat bottom polystyrene titre plates according to Traba and Liang (2011). Evaluation of the inhibitory effects was performed by treating *C. albicans* at adhesion, development, and biofilm maturation stages using a modified time of addition assay by Raut et al. (2013). In brief, 100 µL/well of *C. albicans* (3×10^6 cells/well) in SDB was dispensed aseptically into 96 well-culture plates coated with 50% fetal bovine serum (Sigma Aldrich). For the adhesion stage study, the plate was incubated at 37ºC for 2 h with and without extract. Supernatant then was aspirated, followed by the addition of 100 µL/well of fresh SDB and 50 µl/well MTT in 0.15 M PBS (1:5). After 3 h of incubation under dark condition at room temperature, 50 µL/well of DMSO was added. The absorbance was then measured at 570 nm (Versa Max). For the biofilm development stage, after 2 h of incubation at 37ºC, the supernatant was aspirated and washed by PBS to remove non adhered cells followed by the addition of fresh SDB (100 µl/well) with and without extract and incubation at 37◦C for 24 h. The cell viability was measured by MTT reagent (Sigma Aldrich, St. Louis, America). MTT assay was carried out by monitoring the reduction of MTT after 3 h of incubation under dark conditions. The reduction product was solubilized in DMSO (50 µl /well), and OD was read at 570 nm. The biofilm maturation stage study was carried out by using 24 h old biofilm. Wells with 24 h old biofilm were washed with PBS followed by the addition of 100 µl/well SDB (with or without extract). Cells were further incubated at 37ºC for 48 h, and cell viability was then measured by MTT as described above.

Statistical Analysis

The data were presented as means ± standard error (SE) from three independent experiments. Each test condition was carried out in triplicate. The IC_{50} values of the samples were obtained from at least three determinations. In order to avoid the artefact activity, negative control (medium containing solvent or medium containing extract without *C. albicans*) were also tested.

RESULTS

Figure 1 shows that the n-hexane and ethanol extracts of white (W) and purple (P) varieties of *O. aristatus* leaves (WL, PL) and branches (WB, PB) could inhibit *C. albicans* viability as indicated by lower % relative *C. albicans* cell density as compared to the negative control. Treatment with 3.125 UI/mL nystatin (equal to 0.62 µg/ml) as a positive control, resulted in the lowest *C. albicans* cell viability. The ethanol extracts of both white and purple varieties (WL, WB, PL, PB) exhibited strong cytotoxicity with 50% relative cell density while the n-hexane extracts of WL, WB and PB of *O. aristatus* showed relatively higher % cell viability. Among the extracts tested, the n-hexane extract of PL variety showed the lowest % relative *C. albicans* cell density. The n-hexane extract of *O. aristatus* PL was also observed as the only extract which caused less than 50% of relative *C. albicans* cell density as compared to the negative control. The IC_{50} value of the n-hexane extract of *O. aristatus* PL was 1.3±0.15 mg/mL mg/mL which was higher than that of nystatin (0.07 ± 0.002 µg/mL) and fluconazole (3.36 ± 0.7 µg/mL) as shown in Table 1 respectively.

Table 1. IC_{50} value

Sample	IC_{50} Value (µg/mL)
n-hexane extract PL	1300 ± 150
Nystatin	0.07 ± 0.002
Fluconazole	3.36 ± 0.7

PL, leaves of purple variant

In the biofilm inhibition assay, fluconazole, a widely used anti-fungal drug was used as the positive control (Maubon et al, 2014). The addition of n-hexane extract of *O. aristatus* PL and fluconazole at the adhesion stage showed a significant decrease of *C. albicans* cells compared to untreated cells (Figure 2). The n-hexane extract of *O. aristatus* PL reduced *C. albicans* viability by more than 10% than that of fluconazole at the adhesion stage. The n-hexane extract of *O. aristatus* PL caused 69.2% decrease of *C. albicans* cells, higher than that of fluconazole (54.7%). At the development stage, mild inhibition activity of *C. albicans* growth was observed for both n-hexane extract of *O. aristatus* PL (57.1%) and fluconazole (57.3%). However, both n-hexane extract and fluconazole caused less inhibition to a mature biofilm.

DISCUSSION

The anti-*C. albicans* activity of *O. aristatus* purple and white varieties extracts were evaluated using the crystal violet (CV) viability assay. CV stained the adherent live *C. albicans* cells. Meanwhile, the dead cells of *C. albicans*, following sample treatment, were detached from the culture dish. Low activity of sample against *C. albicans* cells prevented cells from detachment, leading to high levels of CV staining similar to untreated cells (negative control). In this assay nystatin was used as a positive control because of its susceptibility against *C. albicans* (Khan et al., 2018). Screening and IC_{50} evaluation of the ethanol dan n-hexane extracts of purple dan white variants of *O. aristatus* branches and leaves indicated that the n-hexane extract of *O. aristatus* PL showed the strongest cytotoxic effect on *C. abicans* This result suggested that the extract could be a potential source for discovery of lead compound for development of antifungal agent against *C. albicans* infection.

One of the virulence factors of *C. albicans* is the ability to form a biofilm (Lohte et al., 2018). Thus, the effect of n-hexane extract of *O. aristatus* PL against biofilm formation was evaluated at adhesion, development, and maturation stage of biofilm formation. In the biofilm formation assay, fluconazole was used as the positive control to inhibit the adhesion process of *C. albicans*. The n-hexane extract of *O. aristatus* PL showed a similar inhibitory result as fluconazole with pronounced inhibition at the adhesion stage and less activity at the biofilm development and maturation stages, respectively. Fluconazole was reported not only disrupted the membrane and wall integrity but also decreased the incorporation of hyphal-associated wall proteins (As3, hwp1, and P1b5) and wall repaired related protein, which interferes the *C. albicans* adhesion process (Sorgo et al., 2011). In the adhesion process, cell wall proteins such as hwp1 play indirect roles in the adhesion process (McCall et al., 2011).

Neharkar & Laware (2013) reported the antifungal activity of hydro-alcoholic extract of *O. stamineus* against *C. albicans* by using the disk and agar diffusion method. The anti-*C. albicans* activity of the plant was further confirmed by Romulo et al. (2018) who screened the antifungal activity of the ethanol extracts of many Indonesian plants including *O. aristatus* using the broth microdilution method. However, they didn't mention the varieties of tested *Orthosiphon aristatus*. Among the

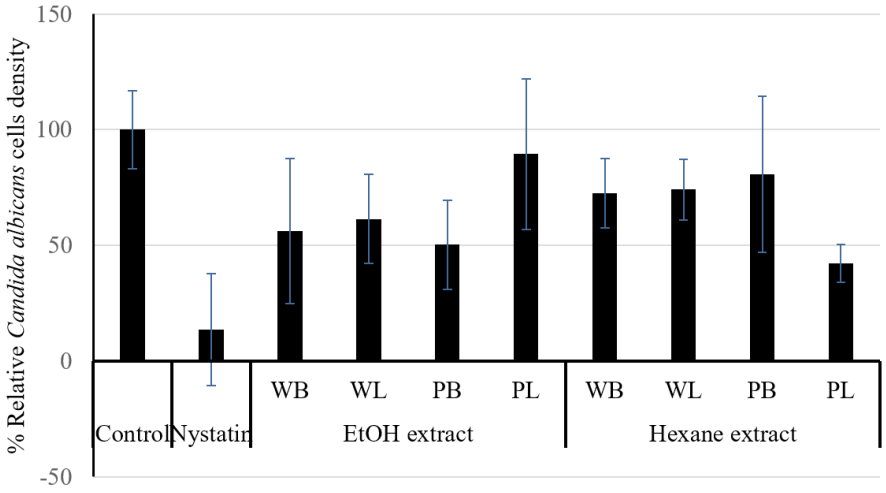

Figure 1. The cytotoxic effect of *O. aristatus* extracts (100 µL/well) against *C. albicans*. Data are represented as the mean ± standard error - from two independent experiments (each in triplicate). Nystatin was tested at 500 UI/ mL. Control, untreated cells; WB, branch of white variant; WL, leaves of white variant; PB, branch of purple variant; PL, leaves of purple variant.

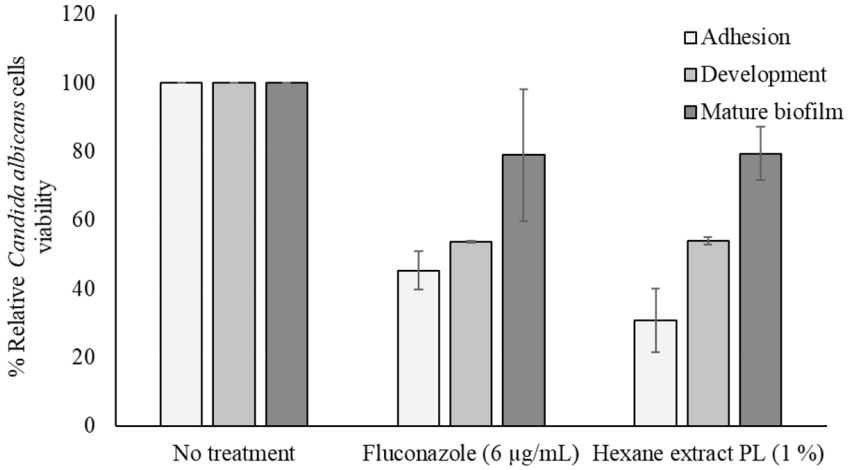

Figure 2. Inhibition of biofilm development of *C. albicans* by the n-hexane extract of leaves of *O. aristatus* purple variant (PL) (2 mg/mL) and fluconazole (6 µg/mL). Data are represented as the mean ± standard error of mean from two independent experiments (each in triplicate).

plants investigated, the extract of *O. aristatus* exhibited the strongest antimicrobial effect, inhibiting the growth of *C. albicans* at MIC value of 128 µg/mL. The active components of *O. aristatus* extract in these studies were polar and possibly phenolic. The anti-fungal action of phenolic compounds have been suggested to be through cell membrane disruption, inhibition of cell wall formation, and inhibition of the mitochondria (Freiesleben & Jager, 2014; Shahzad et al., 2014). In this study, we demonstrated for the first time that the n-hexane and ethanol extracts of white and purple varieties of *O. aristatus,* exhibited inhibitory activity against *C. albicans* due to their cytotoxic and anti-biofilm effects. The results revealed that the n-hexane extract of *O. aristatus* PL possessed the strongest activity against *C. albicans* compared to the other tested extracts. The extract mainly inhibited the adhesion stage of biofilm formation.

The active constituents of the n-hexane extract of *O. aristatus* PL, which were responsible for inhibiting *C. albicans*, have not been identified. The n-hexane extract contained non polar constituents of *O. aristatus* including the essential oils. Hossain *et al.* (2008) identified the chemical composition and anti-fungal effects of the essential oil of *O. stamineus* against various phytopathogenic fungi. The oil was mainly consisted of terpenoids with β-caryophyllene, α-humulene, β-elemene, β-bourbonene and caryophyllene oxide as the major sesquiterpenes while 1-octen-3-ol, β-pinene,

camphene and limonene were the major monoterpenes. Terpenoids have been reported to be able to disrupt the cell membrane, and some of them were also able to destroy the fungal mitochondria (Freiesleben & Jager, 2014). In addition, it was reported that terpenoids potentially inhibited *C. albicans* cells growth at the hyphal form, which is also important for adhesion maintaining process of biofilm formation (McCall et al., 2019; Zore et al, 2011). Thus, it is proposed that active compound(s) of n-hexane extract of *O. aristatus* PL might block the adhesion process by disrupting the hyphal form. However, further evaluation is needed to understand the underlying mechanism.

CONCLUSION

Our present results suggest that the n-hexane extract of leaves of a purple variant of *O. aristatus* has potent inhibitory effects against *C. albicans*. Further studies are necessary to identify the bioactive constituents contributing to the antifungal activity and an indepth study to understand the underlying mechanisms of anti-*C. albicans* effect. The toxicity of the plant extract against human cell line should also be carried out to determine its safety for human use.

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