Dental Journal
Majalah Kedokteran Gigi

Original article

The effect of brotowali (*Tinospora crispa L.*) stem ethanolic extract on the inhibition of *Candida albicans* biofilm formation

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ABSTRACT

Background: *Candida albicans (C. albicans) is an opportunistic pathogen that can be found in the oral cavity and other parts of the body. This species is the main cause of oral candidiasis and forms a biofilm as its virulence factor. Due to increasing cases of anti-fungal resistance, research is needed on methods to control Candida biofilm formation. Brotowali (Tinospora crispa L.) is known to be antifungal, antiseptic and antiparasitic.* **Purpose:** *The purpose of this study is to analyse the ability of brotowali stem extract to inhibit Candida biofilm formation.* **Methods:** *The susceptibility of C. albicans to this extract was examined by a minimum inhibitory concentration (MIC) test using the broth microdilution method. A bacterial adherence assay was performed by similar methods of the MIC assay. A brotowali stem extract of various concentrations were incubated in a yeast peptone dextrose broth medium and stimulated with a C. albicans suspension. The 0.1% crystal violet was used to stain the adherent fungi and measured using a microplate reader at 595 nm. Scanning electron microscopy (SEM) was performed to provide a general overview of the biofilm formation.* **Results:** *The MIC value for the brotowali stem extract was at a concentration of 5,000 µg/ml. Moreover, this extract inhibited fungal adherence starting at a concentration of 250 µg/ml. Observation using SEM confirmed these results. Statistical analysis using one-way analysis of variance demonstrated a significant difference of C. albicans adherence following stimulation with brotowali extract (p < 0.005).* **Conclusion:** *Brotowali stem extract can inhibit C. albicans biofilm formation at an optimal concentration of 1,000 µg/ml.*

Keywords: *adherence; biofilm; C. albicans; Tinospora crispa L.*

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INTRODUCTION

Candida albicans (C. albicans), the most common type of *Candida spp.,* is a normal flora of the human body. These fungi can to turn into opportunistic pathogens that are responsible for mucosal infections, including oral and vaginal.[1,2] *Candida albicans* is the main cause of 95% of oral candidiasis infections.[3] Oral candidiasis can be found on the buccal mucosa, oropharynx and tongue.[4] This species can change into opportunistic pathogens in immunocompromised hosts, patients with catheters, dental prostheses, and those who are taking long-term antibiotics causing superficial candidiasis and systemic candidiasis.[5] The main predisposing factors for developing oral candidiasis are salivary dysfunction, poor denture hygiene, long-term prostheses, topical corticosteroid therapy and smoking.[3]

Candida albicans have distinctive characteristics that include morphological changes and biofilm formation. Its ability to grow into three different morphologies (yeast, pseudohyphae and true hyphae) contributes to biofilm formation,[6] which makes the treatment of *C. albicans* challenging. The National Institute of Health reports that biofilms are responsible for more than 80% of microbial infections.[7] These microbial infections are due to resistance to antifungal agents and increased pathogenicity of *C. albicans.*[8] The biofilm's extracellular matrix plays an essential role in the development of *C. albicans* resistance, as it serves as a physical barrier against drug penetration.[9] In addition, it protects fungi from the innate immune system

by killing neutrophils and monocytes.[10] Previous studies have reported multiple cases of antifungal resistance, including *Candida* resistance to echinocandin drugs, such as micafungin, anidulafungin and caspofungin, and the azole, including fluconazole, itraconazole and isavuconazole.[11,12] Therefore, the utilisation of antifungal agents should be minimised, and methods to control biofilm formation need to be developed.

Indonesia has around 30,000–50,000 species of plants, 7,500 of which have been used as medicinal plants. A well-known medicinal plant, brotowali *(Tinospora crispa L.),* is a wild vine that grows in Asian countries, including Indonesia. Extracts of its stem, root and leaves have been shown to have antifungal properties.[13] More than 65 compounds were identified specifically from brotowali stems, such as flavones, alkaloids, berberine and diterpenes. The antifungal content of brotowali stems is derived from berberine alkaloids and flavonoids.[14] In addition to its antifungal properties, it is analgesic, antipyretic, antiparasitic and antiseptic.[15] This study aims to determine the ability of brotowali stem ethanolic extract to inhibit *C. albicans* biofilm formation.

MATERIALS AND METHODS

Candida albicans was obtained from a patient with candidiasis from Bethesda Hospital, in Yogyakarta, Indonesia. The fungi were isolated and identified using a CHROMagar *Candida* medium (Becton Dickinson, Germany). The colonies of *C. albicans* appeared to be light to medium green (Figure 1). After identification, two colonies of *C. albicans* were grown in a yeast peptone dextrose (YPD) broth medium (Sigma, Missouri, USA) for 24 h at 37°C. The concentration of stock suspension was prepared as a 0.5 McFarland.

The brotowali stem simplicia was obtained and confirmed by a botanical expert at the herbal manufacturing company, CV Merapi Farma, Yogyakarta, Indonesia (ref. 122/MFH-SIMP/IV/2021). The plants used in this study were grown in the village of Hargobinangun, Pakem,

Figure 1.   *Candida albicans* culture in CHROMagar medium.

Sleman, Yogyakarta. The plants were cultivated in appropriate conditions for three months and their brown-black–coloured stems were obtained. The simplicia was processed into an ethanolic extract via a maceration technique (a modification of our previous study[16]) in the biology laboratory of the Faculty of Pharmacy, Universitas Gadjah Mada Yogyakarta, Indonesia. The 1,000 g of simplicia was mixed with 1,000 ml ethanol 96% and stored at 4°C for 24 hours. The macerate was separated by filtration three times and concentrated by a vacuum rotary evaporator at 70°C to obtain an ethanolic extract of brotowali stem. The extract, in paste form, was stored in a refrigerator at 4°C until used. The paste was dissolved in a dimethyl sulfoxide 1% solution (DMSO, Merck, Germany) as a stock in a concentration of 10,000 µg/ml for the analysis of its fungal susceptibility and a concentration of 4,000 µg/ml for the adherence assay. The stock of the extract was filtered using a 0.45 µm syringe filter (Sartorius, Germany) before being diluted with the YPD broth.

The susceptibility of *C. albicans* to the brotowali stem extract was determined via a minimum inhibitory concentration (MIC) test using the broth microdilution method. Ten µl of 0.5 McFarland fungal culture was inoculated into 100 µl of YPD broth containing the brotowali stem ethanolic extract at a concentration ranging from 312.5 to 10,000 µg/ml in a 96-well culture plate (Iwaki, Japan). The experiments were performed by using three well replicates. Fluconazole (Dexa Medica Indonesia) was used as the standard antifungal agent at a concentration of 2,000 µg/ml. The plates were incubated for 24 h at 37°C and observed for the visual absence of turbidity. The MIC of the extract was defined as the lowest concentration that showed no turbidity.[16]

The method used for fungal adherence assay was similar to the susceptibility assay.[17] A 10 µl brotowali stem ethanolic extract at a concentration ranging from 250 to 4,000 µg/ml were added to the 100 µl YPD medium and incubated for 30 min at 37°C in a 96-well culture plate. The culture was stimulated with 10 µl of the 0.5 McFarland *C. albicans* suspension and incubated at 37°C for 24 hours. Following incubation, the YPD medium was removed from each well via washing with a phosphate buffer saline solution (PBS, Sigma-Aldrich, Germany) and fixed with 150 µl absolute methanol for 15 min. The adherent fungi on the wells were stained with 0.1% (wt/vol) crystal violet for 10 min at room temperature and rinsed with PBS twice. The stained, adherent fungi were extracted from the wells using 200 µl of 96% ethanol and transferred to a fresh 96-well plate. The absorbance of the stained adherent bacteria was measured at 595 nm using a microplate reader (Thermo Scientific, USA). The data were analysed via one-way analysis of variance (ANOVA) at a significance level of 0.05 with $p < 0.05$ considered significant. Data analysis was performed via GraphPad Prism (La Jolla, CA, USA).

A general overview of the bacterial adherence to the hydroxyapatite (HA) disks were performed using a scanning electron microscope (SEM). The HA discs

(10mm in diameter and 1.2mm in thickness) were created by placing 500 mg of hydroxyapatite powder into a mould and compressed at 120 Mpa. Finally, the discs were sintered for two hours at 1,300°C and placed in the autoclave for 15 minutes at 100°C to achieve sterilisation.[18]

The HA discs in the 500 µl YPD medium were incubated with 100 µl of the brotowali stem ethanolic extract at a concentration of 0 and 2,000 µg/ml for 30 min at 37°C in a 24-well culture plate (Iwaki, Japan). The culture was inoculated with 100 µl of the 0.5 McFarland *C. albicans* suspension and incubated at 37°C for 24 hours. Thereafter, the discs were rinsed twice with sterile PBS and placed in a primary fixative solution (glutaraldehyde 0.15 M 2.5% [vol/vol] in PBS) for 12 h at 4°C. The discs were rinsed with sterile PBS and treated with the secondary fixative (osmium tetroxide [OsO4 1% w/v]) for 1 hr. The discs were subsequently rinsed with distilled water, dehydrated in an ethanol series (70% for 10 min, 95% for 10 min, and 100% for 20 min) and air dried overnight in a desiccator. The discs were coated twice with platinum vanadium using a sputter ion (Bal-Tec SCD 005; BAL-TEC, Balzers, Liechtenstein) and bonded to carbon double-sided tape for examination via SEM (Hitachi SU3500, Japan).[19]

RESULTS

The MIC value for the brotowali stem extract was at a concentration of 5,000 µg/ml. It was indicated by no turbidity in the YPD medium just as the fluconazole group as a standard of antibacterial agent (Figure 2). The data were representative of three times experiments.

The fungal adherence assay demonstrated that inhibition occurred at concentrations of 500, 1,000, 2,000 and 4,000 µg/ml and were characterised by a decreased optical density (OD) value compared with the control group. The OD value decreased proportionally with increasing extract concentrations, but at a concentration of 4,000 µg/ml, the OD values had values similar to those at a concentration of 2,000 µg/ml. There was no significant difference of the OD value between concentrations of 4,000 µg/ml and 2,000 µg/ml, as seen in Figure 3. The experiment was repeated three times to confirm the data. Statistical analysis via one-way ANOVA showed that there was a significant difference between groups ($p = 0.000$). The aim of this study was to inhibit *C. albicans* biofilm formation, and the optimum concentration of brotowali stem deletedextract was found to be 1,000 µg/ml.
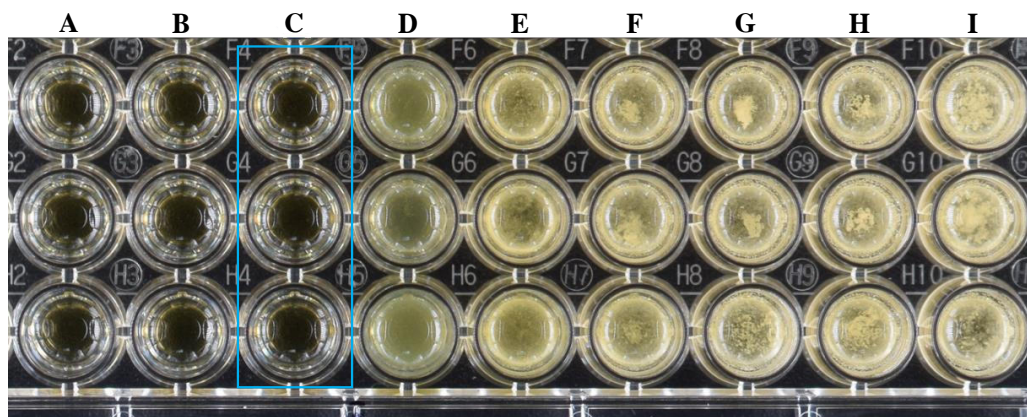
Figure 2. The fungal susceptibility. No treatment (I); the concentration ranging from 10,000 µg/ml (B) to 156.25 µg/ml (H). The MIC was at a concentration of 5,000 µg/ml (C, blue box); Fluconazole used as a standard of antifungal agent (A).
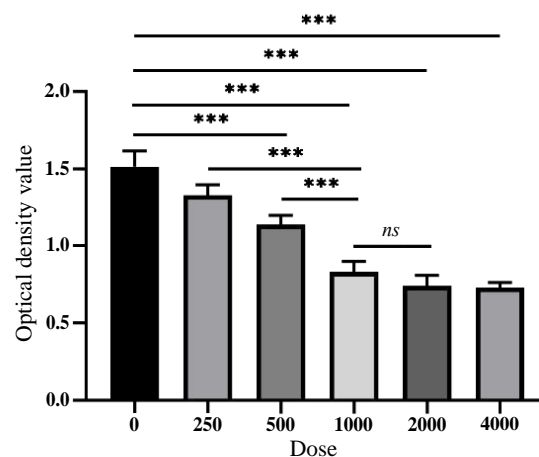
Figure 3. Optical density values of *C. albicans* adherence at various concentrations. Notes: *ns*: not significant; ***: $p < 0.001$.
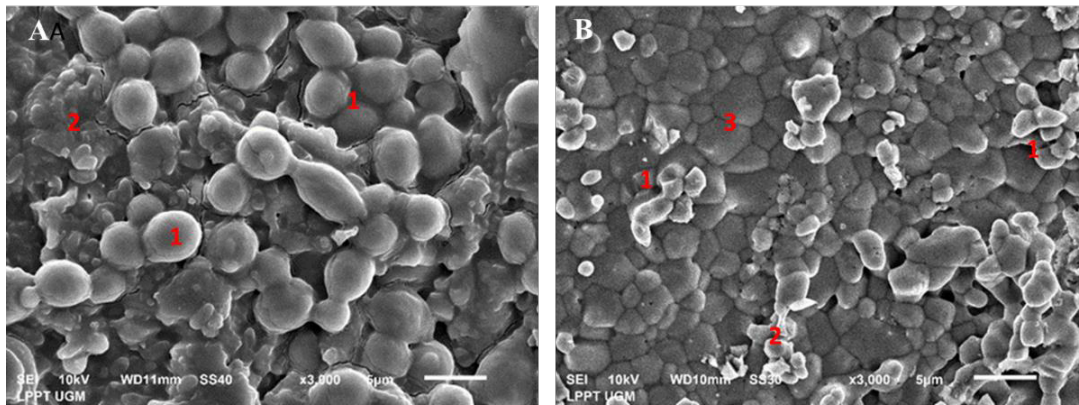
Figure 4.   Scanning electron microscopy of HA surface. The number of *C. albicans* colonies (1) were higher in the untreated disk (A) compared with the 1,000 μg/ml extract-treated disk (B). The extracellular matrix (2) of the *C. albicans* biofilm was detected in a higher intensity on the HA crystal (3) of the untreated disk (magnification 3,000×).

Observations via SEM confirmed these results as representative of the experimental groups. Yeast cells can be observed on both the untreated and extract-treated disks. The number of *C. albicans* colonies were higher in the untreated disk compared with the 1,000 μg/ml extract-treated disk. The extracellular matrix of *C. albicans* biofilm was also detected in a higher intensity on the untreated group (Figure 4).

DISCUSSION

The ethanolic extract of brotowali stem *(Tinospora crispa L.)* showed antifungal activity against *C. albicans* starting at a concentration of 500 μg/ml. The MIC value for the brotowali stem extract was at a concentration of 5,000 μg/ml. The antifungal activity of the brotowali stem *(Tinospora crispa L.)* occurs due to its flavonoids and berberine alkaloids. So far, there has been little research on brotowali stem extract's antifungal properties. A previous study conducted by Warsinah et al.[20] using a disk diffusion method showed antifungal activity of this extract against *C. albicans* and also reported that the ethanol fraction had the best inhibition due to the ethanol's ability to extract plant antifungal compounds, such as phenols, fatty acid groups and terpenoids compared with the chloroform, ethyl acetate, n-hexane and distilled water fractions.[20] Therefore, in this study, ethanol was chosen as the solvent.

Many factors contribute to the *Candida* biofilm formation process. The initial step involves the attachment of the hyphae to a surface. The hyphal cells then proliferate to form microcolonies, forming the biofilm base layer. Morphological changes occur, followed by the secretion of extracellular polymeric substances and the formation of an extracellular matrix.[10,21,22] The attachment of *C. albicans* to the buccal epithelium and mucosa is mediated by various protein molecules, such as *ALS* and *HWP1*, which are dominantly expressed.[23,24] The antifungal effect of brotowali stem *(Tinospora crispa L.)* on *C. albicans* biofilms is influenced by berberine alkaloids and flavonoids.

Berberine is a natural isoquinoline alkaloid found in herbal plants and distributed in the roots, bark and stem of the plant.[25] This substance has a yellow color and bitter taste. Berberine and its derivatives have antifungal, antibacterial and anti-inflammatory potential.[24] Berberine affects biofilm formation by reducing the thickness of the biofilm and destroying its structure. Berberine is able to suppress the dimorphic changes of *C. albicans,* thus inhibiting the development of biofilms.[23] The mechanism of berberine as an antifungal agent occurs via the reduction of regulatory gene expression in biofilm formation and proliferation of hyphae. Berberine causes changes in the mitochondrial membrane potential (Δψm). The role of mitochondria in fungi is essential in signaling the metabolic pathway during virulence and infection in the host and defense against oxidative stress.[23] Changes in the mitochondrial membrane potential opens pores in the membrane, triggering the release of proapoptotic factors and resulting in fungal apoptosis.[26] In addition, the combination of berberine and fluconazole accumulates mainly in the nucleus of *C. albicans* and cause DNA damage.[27]

Flavonoids are secondary metabolites and the largest class of polyphenols widely found in plants.[26] The antifungal activities of flavonoid occurs via several mechanisms, such as plasma membrane disruption, induction of mitochondrial dysfunction, inhibition of cell formation, protein synthesis and efflux pumps.[28] Flavonoids such as quercetin can increase the production of farnesol, which suppresses biofilm formation and hyphae development in *C. albicans.*[22] The combination of the flavonoid quercetin with fluconazole has the ability to prevent cell-to-cell communication, thereby disrupting gene expression in biofilm formation. In this study, it was hypothesised that alkaloid, berberine and flavonoid compounds in the brotowali stems inhibited fungal growth and the biofilm formation.

In this study, an HA disk was used as a medium for *C. albicans* adherence. However, these results had not yet represented the *C. albicans* adherence to mucosal surface. Future studies are recommended to analyse the ability of this extract to inhibit *C. albicans* adherence on mucosal

Hutomo et al./Dent. J. (Majalah Kedokteran Gigi) 2022 March; 55(1): 21–25

25

epithelium. In conclusion, brotowali *(Tinospora crispa L.)* stems were found to have antifungal activity against *C. albicans.* We suggest that this extract may serve as a potential natural product for oral care medicaments to control *Candida* biofilm formation, particularly in immunocompromised hosts.

ACKNOWLEDGEMENT

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