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EFFECT OF MORINGA (*Moringa oleifera)* LEAF EXTRACT ON *IN VITRO* INHIBITION OF *Candida albicans* BIOFILM

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ABSTRACT
Background: *Oral candidiasis is an opportunistic infection in the oral cavity. This infection is caused by the fungus Candida albicans. The most important virulent attribute of this fungus is its ability to form biofilms, which can adhere to mucosa, epithelial lining, organs, prostheses or dentures. The formed biofilm is resistant to antifungal drugs.* ***Objectives:*** *The purpose of this study was to determine the effect of Moringa (Moringa oleifera) leaf extract on the inhibition of Candida albicans biofilm formation in vitro.* ***Methods:*** *This type of research is experimental study using post tests with control group design. Fungus used in this study was Candida albicans ATCC 10231. Determination of minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) were conducted by microdilution method. Inhibition test of C. albicans biofilm formation was carried out using the polystyrene microplate assay method. Concentration of extracts used in this study were 12.5%, 6.25%, 3.13% and 1.57%. Inhibitory activity of biofilm was measured using a crystal violet (CV) assay.* ***Results:*** *The results showed that MIC of Moringa leaf extract against the fungus C. albicans was at a concentration of 6.25%. Moringa leaf extract starting at a concentration of 3.13% already has had ability to inhibit formation of C. albicans biofilm.* ***Conclusion:*** *It can be concluded that Moringa leaf extract can inhibit formation of Candida albicans biofilm, so it can be developed as an alternative herbal ingredient to prevent oral candidiasis.*

Keywords: *Biofilm, Candida albicans, Moringa Leaf Extract*
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INTRODUCTION

Oral candidiasis is an opportunistic infection in the oral cavity, which is mainly caused by *Candida albicans*.[1] Main complaint of oral candidiasis patients are mostly pain or soreness in the oral cavity (57.1%), burning sensation in the oral cavity (4.1%), painful swallowing (6.1%), a combination of pain or stinging with heat (26.6%). The most common form of candidiasis lesions found is a white pseudomembranous plaque and the most common location is dorsum of tongue.[2] *Candida albicans* is the most common fungus found in the human oral cavity. The prevalence of Candida albicans isolated from oral cavity of healthy general population is around 30%-50% and can turn into a pathogen if there is a change in the oral cavity biology, both changes in temperature and *pH*.[1] These two indicators determine imbalance of oral pathogen.[3] *Candida albicans* is the most virulent and pathogenic species in humans, followed by *C. tropicalis*. Both of these species have ability to form biofilms that cause infectious diseases in the oral cavity.[4] *C. albicans* is one of fungi that can cause disease in humans. The most important virulent attribute of this fungus is its ability to form biofilms, which can adhere to mucosa, epithelial lining, organs, prostheses or dentures. The biofilm formed is resistant to antifungal drugs.[5] Therefore, research is needed to develop alternative materials that can inhibit formation of biofilms as a strategy to prevent *oral candidiasis*.

The Indonesian government 2007 has directed its attention to the development of medical services especially alternative complementary medication in health care facilities. This is indicated by the existence of a policy to develop Indonesian herbal medicines through various steps by issuing the Minister of Health Regulation No. 1109/Menkes/PER/IX/2007 concerning complementary and alternative medicine in health care facilities.[6] Nowadays, people are starting to turn their attention back to nature, such as the effort to use natural ingredients as medicine in health care

programs. People return to using natural ingredients because of their belief that natural ingredients have fewer side effects compared to modern or synthetic drugs.

Moringa is a plant that has many health benefits. Almost all parts of this plant can be utilized, such as leaves, roots, seeds, bark, fruit, and flowers. Moringa has many benefits, including anti-tumor, antipyretic, anti-inflammatory, anti-ulcer, anti-hypertensive, anti-oxidant, anti-bacterial, and anti-fungal.[7] Moringa leaves contain phenols (0.19%), alkaloids (0.42%), tannins (8.22%), and saponins (1.75%).[8] These ingredients are useful as anti-bacterial and anti-fungal. Moringa leaves extracted using ethanol and ethyl acetate can inhibit the growth of *Staphylococcus aureus, Escherichia coli, Salmonella typhi*, and *Candida albicans*.[8] The polyphenol content in certain parts of a plant varies. The highest polyphenol content in leaves, especially during photosynthesis, continues in stems, seeds, and roots.[9] Phenols, especially flavonoids, can inhibit the work of fungi by denaturing proteins, causing damage to cell membranes. This damage can cause fungal cell death.[10,11]

Although several studies on the benefits of Moringa leaves as herbal ingredients have been carried out, research on the benefits of Moringa leaves as an antifungal has not been widely studied, especially related to the inhibitory effect on oral biofilm formation. The purpose of this study was to determine the effect of Moringa (*Moringa oleifera)* leaf extract on the inhibition of *Candida albicans* biofilm formation in vitro.

METHODS

The type of research is an experimental study using post-test design with a control group design to determine concentration on inhibition of *C. albicans* biofilm formation. Moringa leaves are collected from the Moringa plant that grows in the areas of Purwosari, Sinduadi, Mlati, Sleman. Plant identification was carried out at the Plant Systematics Laboratory, Faculty of Biology, Universitas Gadjah Mada, Yogyakarta. This research has received an ethical clearance number e-KEPK/Polkesyo/0359/III/2021.

Extract preparation

Making simplicia was carried out by means of fresh Moringa leaves, washed with running water, then dried in an oven (temperature of 50°C), for 48 hours. After drying, Moringa leaves were ground into powder.

Extracts were made by maceration method using 96% ethanol as solvent. The ratio of powder and solvent is 1:7. The immersion was carried out for 24 hours, then filtered, the filtrate was evaporated at a temperature of 60°C. The weight of the extract obtained was 42.6 gr.

The mother extract solution was prepared by dissolving Moringa leaf extract with PBS solvent, by dissolving 10 grams of extract in 10 ml of PBS. Various concentrations (50%, 25%, 12.5%, 6.25%, 3.13%, and 1.57%) of Moringa leaf extract were used in this study. Then the pH of the extract was measured, the mother extract solution was then filtered using a filter with a diameter of 0.45 µm and 0.22 µm.

Microbiological test

The microbiological test was carried out at the Integrated Research Laboratory, Faculty of Dentistry, Universitas Gadjah Mada, Yogyakarta. Confirmation of *Candida albicans* was conducted using CHROMagar selective media. *Candida albicans* ATCC 10231 was cultured on SDA medium for 24 hours at 37°C. Then one colony was inoculated in 5 ml of SDB media and incubated for 24 hours and equalized with Mc. Farland 0.5 (equivalent to 1.5 X 10^8 CFU/mL), then diluted to obtain 1 X 10^7 CFU/mL.

Determination of MIC and MBC by microdilution method[12], using a 96-well flat-bottom microplate in various concentrations (50%, 25%, 12.5%, 6.25%, 3.13%, 1.57%). The MIC test was carried out by preparing 8 wells, 6 wells for the treatment test, 1 well for the positive control (the extract was replaced with Nystatin), and 1 well for the negative control (the extract was replaced with PBS). In the first treatment well, 50 µL of sterile SDB was added to 50 µL of the mother extract solution, and mixed, then 50 µL was taken and transferred to the second well, and so on until the eighth well. From this last treatment well, 50 µL was taken and discarded so each well contains 50 µL. Then 40 µL of SDB and 10 µL of C. albicans suspension were added to each well. Furthermore, it was incubated for 24 hours at 37°C. MIC was indicated by the absence of turbidity after incubation and absorbance were measured using a microplate reader. The MBC test was carried out by growing of 100 µL of culture from wells showing MIC and cultures with exact concentrations above it, on SDA media. Then incubated at 37°C for 24 hours. MBC was indicated by the absence of fungal colonies growing in the media. The MIC and MBC determination tests were repeated three times.

An inhibition test for *C. albicans* biofilm formation was carried out using the polystyrene microplate assay method in various concentrations (50%, 25%, 12.5%, 6.25%, 3.13%, 1.57%) was determined as described previously by Weerasekera with modifications.[4] A total of 100 µL of *C. albicans* suspension solution (1 X 10^7 CFU/mL in SDB) was incubated for 90 minutes at 37°C. Then the media was aspirated and rinsed with sterile PBS 2 times. Next, 50 µL of SDB and 50 µL of the extract with various concentrations were added in 4 wells, one well was added 50 µL of SDB and 50 µL of PBS, and the next well was

added 50 µL of SDB and 50 µL of Nystatin, subsequently were incubated for 24 hours at 37°C. The inhibitory activity of the biofilm was measured using a crystal violet (CV) assay, by aspirating the solution in a well, then was added by 100 µL of 1% CV solution and incubated for 20 minutes at 37°C, afterward was rinsed twice with sterile PBS. Later on, it was discolored by adding 200 µL of 96% of ethanol. Then 100 µL of ethanol was transferred to a new well and absorbance was measured at 595 nm using a microplate reader. This test was repeated three times.

Statistical analysis

To analyze the effect of various concentrations of Moringa leaf extract in the inhibition of Candida albicans biofilm formation, a one-way analysis of variance was used, followed by the *Post Hoc LSD test*.

RESULTS

Plant identification resulted that:

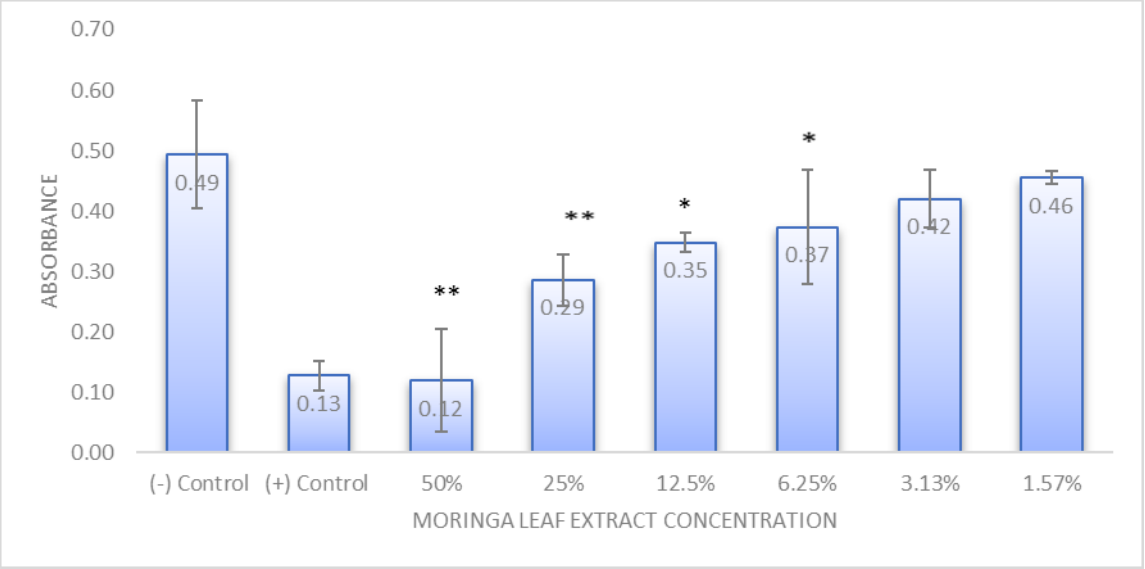
Division	: Tracheophyta
Sub Division	: Spermatophyta
Class	: Magnoliopsida
Super Order	: Rosanae
Order	: Brassicales
Family	: Moringaceae
Genus	: Moringa
Species	: *Moringa oleifera L.*

Candida albicans ATCC 10231 was obtained from the Integrated Research Laboratory, Faculty of Dentistry, Universitas Gadjah Mada, Yogyakarta. Prior to use, confirmation was carried out using CHROMagar selective media. The results can be seen in Figure 1.

Figure 1. *C. albicans* colonies in media of CHROMagar (round and green in color)

From Figure 1, it can be seen that the growth of colonies is round and green in color. This is a characteristic of *C. albicans* when they were grown on CHROMagar selective media, so it can be concluded that the fungus planted is *C. albicans.* Furthermore, the fungus was used in this study.

The minimal inhibitory concentration (MIC) test of Moringa leaf extract (*Moringa oleifera*) against *Candida albicans* was carried out using the microdilution method using Moringa leaf extract in various concentrations (50%, 25%, 12.5%, 6.25%, 3.13%, 1.57%). The results of the absorbance measurement using a microplate reader can be seen in Figure 2.

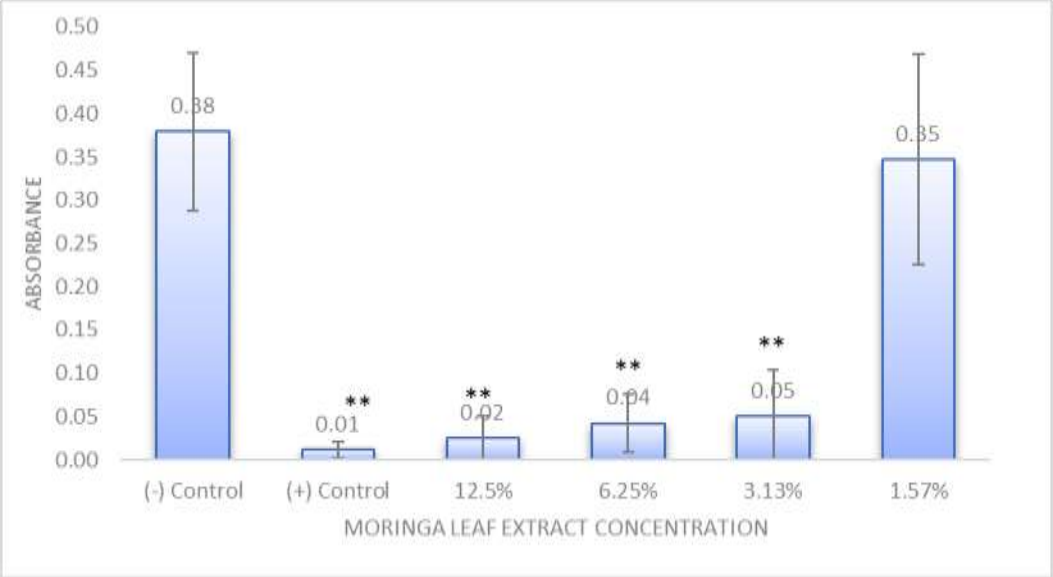


** Sig. p<0.01, * Sig. p<0.05, MIC at 6.25% concentration, Control +: Nystatin, Control -: PBS

Figure 2. Inhibition of Moringa leaf extract against *C. albicans*

Figure 2. shows the inhibition of the growth of *C. albicans* after exposure to Moringa leaf extract. Seen at a concentration of 6.25% there was a decrease in absorbance when compared to the negative control group. After the significance test was carried out, the Moringa leaf extract starting at a concentration of 6.25% showed significant inhibition of the growth of the fungus *C. albicans*, so this concentration was determined as the Minimal Inhibitory Concentration (MIC) of Moringa leaf extract against *C. albicans*.

The inhibition test of *C. albicans biofilm* formation used the *polystyrene microplate assay method.* The concentration of extracts used in this study were 12.5%, 6.25%, 3.13% and 1.57%. The inhibitory activity of biofilm was measured using a crystal violet (CV) assay. The results of the potential test of Moringa leaf extract in inhibiting the formation of *C. albicans biofilm* can be seen in Figure 3.



** Sig. p < 0.01, the inhibitory potency against biofilm starts at a concentration of 3.13%

Figure 3. The potency of Moringa leaf extract for inhibiting the formation of *C. albicans* biofilm

From Figure 3, it can be seen that the absorbance concentration of Moringa leaf extract was 3.13% lower than the absorbance in the negative control group. This indicates that the Moringa leaf extract starting at a concentration of 3.13% already has the ability to inhibit the formation of *C. albicans biofilm*.

DISCUSSION

Our results showed that Moringa leaf extract could significantly inhibit the growth of the fungus *C. albicans ATCC* 10231 at a concentration of 6.25% (MIC). This is probably due to the presence of flavonoids. This research is in line with Nuryanti's research, that Moringa fruit extract could inhibit the growth of *Candida albicans*.[13] Based on her research showed that Moringa fruit contains flavonoids. Flavonoids can inhibit fungal growth because they cause protein denaturation resulting in an increase in permeability of fungal cell membrane. The increase in membrane permeability causes damage to fungal cells which causes death in the fungus.[13] According to Yang, damage to the wall and membrane of *C. albicans* was probably caused by flavonoid baicalein. This resulted in *C. albicans* not being able to maintain its morphological structure and its exchange components intracellularly and extracellularly. In addition, many enzymes in cell membrane experience dysfunction so that nutrients that can be absorbed by *C. albicans* and synthesis of macromolecules decreases, which will end in apoptosis until death of *C. albicans*.[10] This study is also in line with study of Serpa et al. who examined the antifungal activity of flavonoid baicalein against Candida species. The results of his research indicated that the flavonoid baicalein inhibited growth and decreased cell viability of *C. albicans*, *C. tropicalis* and *C. parapsilosis*.[14]

Inhibition of biofilm formation occurred at a concentration of 3.13% below the MIC. This demonstrates that the inhibition of biofilm formation is not only caused by the inhibition of growth of the fungus (*C. albicans)* but there are other factors that cause changes in virulence factors of *C. albicans* in forming biofilms. Formation of *C. albicans* biofilm depends on the attachment of yeast cells to a surface followed by attachment between yeast cells. Hyphae formation is main component of *C. albicans* biofilm formation.[15] The

possibility of secondary metabolites produced by Moringa leaves can reduce the ability of *C. albicans* to form hyphae so that no biofilm is formed. Another possibility is that Moringa leaf extract can inhibit the attachment of yeast cells to the surface so that biofilms cannot be formed. This is in line with research of Ivanov et al. which showed that flavonoids have moderate activity in inhibiting growth of *C. albicans* hypha. Among flavonoid groups studied, apigenin, apigetrin and isoquercitrin showed the strongest ability to inhibit hypha formation.[16]

Another possibility is that Moringa leaf extract can inhibit the attachment of yeast cells to the surface so that biofilms cannot be formed. This is consistent with a study conducted by Onsare and Arora who examined anti-biofilm potential of flavonoids extracted from Moringa seed coats against *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Candida albicans*. Their research showed that flavonoids can inhibit cell attachment and interfere with biofilm formation and biofilm metabolic activity.[17]

From the results of this study, the exact mechanism of inhibition of *C. albicans* biofilm formation is not known, so further research is needed to determine the mechanism of inhibition of *C. albicans* biofilm formation by Moringa leaf extract. In addition, other research is required to determine the content of Moringa leaf extract which has a potency to inhibit formation of *C. albicans* biofilms. This is in line with the research of Ivanov et al. which showed that flavonoids have moderate activity in inhibiting the growth of *C. albicans* hypha. Among the flavonoid groups studied, apigenin, apigetrin and isoquercitrin demonstrated the strongest ability to inhibit hypha formation.[16]

Based on the results of this study, it can be concluded that Moringa leaf extract can inhibit the formation of *Candida albicans biofilm* at a concentration 3.13%, therefore it can be developed as an alternative herbal ingredient to prevent oral candidiasis.

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