Can Moringa oleifera Leaf Ethyl Acetate Extract Inhibit Candida albicans Planktonic Cell Growth and Biofilm Formation In Vitro?

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Abstract

Introduction: Candida albicans (C. albicans) is the most common fungus found in the human oral cavity. This fungus has the ability to form a biofilm that causes infectious diseases in the oral cavity. Nowadays, the incidence of infectious diseases caused by C. albicans was increasing due to resistance to antifungal drugs. This study aimed to investigate the effect of Moringa oleifera ethyl acetate extract on the inhibition of C. albicans planktonic cell growth and biofilm formation in vitro. Methods: C. albicans (ATCC 10231) was the fungus used in this study. Determination of inhibition planktonic cell growth by microdilution method. The polysaccharide microplate assay method was used to test the inhibition of C. albicans biofilm formation. The extract concentrations used in this study were 25%, 12.5%, 6.25%, 3.13%, and 1.57%, respectively. A crystal violet (CV) assay assessed the biofilm's inhibition of planktonic cell growth and biofilm formation in vitro. Results: The minimal inhibitory concentration of Moringa oleifera leaf ethyl acetate extract against the planktonic form of C. albicans was found to be 1.57%. Starting at 6.25% concentration, Moringa leaf ethyl acetate extract inhibits the formation of C. albicans biofilm. Conclusion: Since Moringa oleifera leaf ethyl acetate extract inhibits C. albicans planktonic and biofilm formation, it has the potential to be developed as an alternative antifungal agent. Keywords: Moringa leaf extract, planktonic cells, biofilm, Candida albicans

INTRODUCTION

The most common fungus in the human oral cavity is C. albicans. This fungus is commensal in healthy people, but it can be a pathogen if the oral cavity biology was changed. C. albicans is the most abundant species worldwide, accounting for 66% of all Candida species. The prevalence of C. albicans as the dominant species has increased from 37% in Latin America to 70% in Norway as a result of rising candidiasis incidence, increased populations of susceptible individuals, and treatment delays caused by antifungal drug resistance.

In humans, C. albicans is the most virulent and pathogenic species. This species can produce biofilms that cause infectious diseases in the oral cavity. According to the research, C. albicans is one of the fungi that can cause disease in humans. The ability of this fungus to form biofilms is its most virulent aspect. Antifungal drugs are not effective against the biofilm that has formed. As a result, research is needed to develop alternative materials that can inhibit the formation of biofilms as a strategy for developing anti-fungal agents.

Moringa oleifera is a plant that originated in the southern Himalayan hills. Moringa is a long-lived plant that can reach a height of 7-12 meters. The leaves are long-stemmed compound leaves with alternating leaf arrangements. Moringa leaves are light green when young, but as they mature, the green color darkens. Moringa is a plant that has numerous health benefits, including anti-cancer, anti-inflammatory, anti-oxidant, anti-microbial, and immune response. By denaturing proteins and causing cell membrane damage, phenols, particularly flavonoids, can inhibit fungi activity. This harm has the potential to kill fungal cells.

In carrying out the extraction, the right solvent is needed to get the optimal extract. Factors that need to be considered in the selection of solvents include selectivity, toxicity, polarity, and ease of evaporation. Ethyl acetate is a solvent with low toxicity that is semi-polar, therefore it is expected to attract polar and non-polar compounds from Moringa leaves. Although studies on the advantages of moringa leaves as herbal constituents have been conducted, there has not been much research on the advantages of moringa leaves as an antifungal, notably the inhibitory effect on the production of biofilms. The aim of this study was to examine the effects of ethyl acetate leaf extract from Moringa oleifera on C. albicans' planktonic cell growth and biofilm formation in vitro.

MATERIALS AND METHODS

Plant Collection and Extraction

The Moringa plant is harvested for its leaves in the Purwosari, Sinduadi, Mlati, Sleman regions, in Indonesia. The Plant Systematics Laboratory, Faculty of Biology, Universitas Gadjah Mada, Yogyakarta, conducted plant identification. The
extraction of the secondary metabolite from Moringa leaves was carried out according to with slight modifications. After cleaning under running water, the moringa leaves dried in an oven for 48 h at 50°C. Then, turned into powder after drying. The powder was mixed with ethyl acetate in the ratio 1:7 (w/v), for 24 h, after which the filtrate was filtered and concentrated at 60°C. The extracted substance weighed 20.1 gr. The basic extract solution was made by dissolving 5 grams of Moringa leaf extract in 5 ml of 2% DMSO. After measuring the extract’s pH, the solution was filtered.

**Determination of Inhibition Planktonic Cell Growth**

To determine the concentration of moringa leaf extract that can inhibit planktonic cell growth using the Minimum Inhibitory Concentration (MIC) test. The test was performed at the Integrated Research Laboratory, Faculty of Dentistry, Universitas Gadjah Mada, Yogyakarta. The initial inoculum of *C. albicans* (ATCC 10231) was inoculated into 5 ml of Sabouraud Dextrose Broth (SDB) medium, incubated for 24 h at 37°C, and adjusted to 0.5 Mc. Farland standard.

The MIC of moringa leaf extract was determined by the microdilution method. The different concentrations of moringa leaf extract were plated on a 96-well flat-bottom microplate, including the positive control (Nystatin), and the negative control (Phosphate Buffer Saline = PBS). Furthermore, the fungal suspension was inoculated to the well that contains various concentrations of extract and control to obtain the final concentration of *C. albicans* 1 x 10^6 CFU/mL. Then it was incubated for 24 h at 37°C in aerobic condition. MIC was indicated by the absence of turbidity after incubation and the absorbance was measured at 595 nm using a microplate reader. The experiment was performed in triplicate.

**Determination of Inhibition Biofilm Formation**

The inhibition of *C. albicans* biofilm formation was performed using a polystyrene microplate assay method according to with modification. In the first, 100 µL/well standard *C. Albicans* suspension was inoculated in triplicate into wells of a sterile flat-bottomed microtiter plate and incubated for 90 minutes at 37°C for initial adhesion. Following a 90-minute incubation period, the plate was carefully washed twice with 200 µL of sterile phosphate-buffered saline (PBS).

Furthermore, 50 µL SDB and 50 µL extracts of various concentrations of Moringa leaf extract or positive and negative control were added, then the plate was incubated for 24 hours at 37°C. The inhibitory activity of the biofilm formation was measured using a crystal violet (CV) assay.

For the CV assay, after incubating, the non-adherent cell was aspirated. A 100 µL of 1% CV solution was added to each well and incubated for 20 minutes at 37°C before the plate was carefully washed twice with sterile PBS. Finally, CV-stained cells were decolorized by adding 200 µL of 95% ethanol per well. The absorbance at 595 nm was measured using a microtiter plate reader after 100 µL of ethanol was transferred to a new microtiter plate.

**Statistical analysis**

A one-way analysis of variance was used to analyze the effect of various concentrations of Moringa leaf extract in inhibiting planktonic cells of *C. albicans* growth and biofilm formation, followed by the Post Hoc LSD test.

**RESULTS**

a. The result of plant identification:

<table>
<thead>
<tr>
<th>Division</th>
<th>Tracheophyta</th>
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</thead>
<tbody>
<tr>
<td>Sub Division</td>
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<td>Super Order</td>
<td>Rosaneae</td>
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<td>Order</td>
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<td>Family</td>
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</tr>
<tr>
<td>Species</td>
<td>Moringa oleifera Lam.</td>
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</tbody>
</table>

b. Results of ethyl acetate extract of Moringa leaf against *C. albicans* in inhibiting planktonic cell growth.

The microdilution method was used to conduct the inhibiting planktonic cell growth using different concentrations of Moringa leaf ethyl acetate extract (25%, 12.5%, 6.25%, 3.13%, 1.57%, and 0.78%). Figure 1 displays the outcomes of the microplate reader-based absorbance measurement.

![Figure 1: Inhibition of Moringa leaf ethyl acetate extract against *C. albicans*](image)

**Figure 1**: Inhibition of Moringa leaf ethyl acetate extract against *C. albicans*

According to our findings that the growth of *C. albicans* could be greatly inhibited by an ethyl acetate extract of Moringa leaf at a concentration of 1.57% (MIC).

c. Results of ethyl acetate extract of Moringa leaf against *Candida albicans* in inhibiting biofilm formation.

The polystyrene microplate assay method was used to study the formation of *C. albicans* biofilms. The study used extract concentrations of 25%, 12.5%, 6.25%, and 3.13%. A crystal violet (CV) assay was assessed to determine the biofilm’s inhibitory activity. Figure 2 shows the potential of Moringa leaf ethyl acetate extract in inhibiting a biofilm of *C. albicans*.
Alkaloids, like flavonoids, have antifungal properties. Alkaloids and tannins, saponins, and flavonoids. Flavonoids are the largest group of phenolic compounds, which have effective properties against bacteria, viruses, and fungi. This study supports work, which found that a moringa fruit extract can inhibit the growth of C. albicans. The research demonstrates that flavonoids are present in the fruit of the moringa plant. Because they cause protein denaturation and enhance the permeability of fungal cell membranes, flavonoids can limit the growth of fungi. This increase in membrane permeability causes damage to fungal cells which causes death in fungi. According to Dewi, flavonoids have the potential to serve as antifungals because they include phenolic compounds that can combine with ergosterol to generate complex molecules in fungal cell membranes. This phenol and ergosterol compound make fungal cells’ pores larger, which allows tiny molecules like nucleic acids and proteins to exit the cells and induce death. This study is also consistent with the work, which investigated the flavonoid baicalein’s antifungal properties against various Candida species. The findings demonstrated that C. albicans C. tropicalis and C. parapsilosis cell viability and proliferation were both suppressed by the flavonoid baicalein.

Alkaloids, like flavonoids, have antifungal properties. Alkaloids are semipolar metabolites. The mechanism of alkaloids as antifungals is to insert them between the cell wall and DNA, preventing fungal DNA replication and thus disrupting fungal growth. Moringa leaf ethyl acetate extract can attract tannin compounds. Tannins are included in the non-polar polyphenol group. The antifungal mechanism possessed by tannins is their ability to inhibit the synthesis of chitin which is used for the formation of cell walls in fungi and damage cell membranes, hence that fungal growth is inhibited. Tannins are lipophilic compounds that are easily attached to fungal cell walls. Saponins are also compounds that can be extracted with ethyl acetate as a solvent. Saponins have a glycosyl that functions as a polar group and a steroid group as a non-polar group. The mechanism of saponins as antifungals is by disrupting the stability of cell membranes, resulting in the lysis of microbial cells.

Figure 2 showed that ethyl acetate extract of Moringa leaf at a concentration of 6.25% can inhibit the formation of C. albicans biofilm. According to the formation of C. albicans biofilms is dependent on yeast cell attachment to a surface, followed by yeast cell attachment to each other. Hyphae formation is the main component of C. albicans biofilm formation. Secondary metabolites produced by ethyl acetate extract of Moringa leaf have the potential to reduce C. albicans to form hypha, preventing the formation of biofilm. This is consistent with the study of, which showed that flavonoids had a modest inhibitory effect on the growth of C. albicans hypha. Apigenin, apigetrin, and isouqueritrin molecules demonstrated the highest capacity to suppress hypha development among the examined flavonoid groupings. Another theory is that yeast cells may be prevented from adhering to surfaces by the ethyl acetate extract of Moringa leaf, preventing biofilm formation. This is consistent with research done by, who examined the effectiveness of flavonoids derived from Moringa seed shells against Staphylococcus aureus, Pseudomonas aeruginosa, and Candida albicans. The results showed flavonoids can inhibit cell attachment and interfere with biofilm formation and biofilm metabolic activity.

Because the mechanism of inhibition of C. albicans biofilm formation by ethyl acetate extract of Moringa leaf is still unknown, more research is required. Furthermore, more research is needed to determine the compound of Moringa leaf ethyl acetate extract, which has the potential to inhibit the formation of C. albicans biofilm.

CONCLUSION
Based on this study, it can be concluded that since Moringa oleifera leaf ethyl acetate extract inhibits C. Albicans planktonic growth and biofilm formation, it has the potential to be developed as an alternative anti-fungal agent.

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CONFLICT OF INTEREST
The author declared that there is no conflict of interest.

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