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Potential of *Moringa oleifera* (*M. oleifera*) Leaf Extract as an Antiseptic against *Candida albicans* using Percentage Kill Test

Conny Riana Tjampakasari[1*], Ariyani Kiranasari[1], R. Muh. Kevin Baswara[2]
[1] Department of Clinical Microbiology, Faculty of Medicine. Universitas Indonesia, Jakarta Pusat, Indonesia
[2] Medical Study Program, Faculty of Medicine, Universitas Indonesia, Jakarta Pusat, Indonesia

ABSTRACT

Candida albicans is a commensal microorganism that is commonly found as a normal flora in the body. However, impaired immune conditions can cause this fungus to turn into a pathogen. This Microorganism is one of the causes of mortality and morbidity in the world. The use of antiseptics is beneficial for the prevention and treatment of fungal infections. *Moringa oleifera* is a plant that is often found in Asia and Africa, has various bioactive components that have the potential to be antiseptic. Until now, there has been no research on the antiseptic potential of *M. oleifera* against *C. albicans.* Therefore, the study was conducted to test the activity of *Moringa* leaf extract (*M. oleifera*) as an antiseptic against *C. albicans.* This experiment was conducted using the percentage kill method to determine the potential activity of the extract as an antiseptic against *C. albicans.* The comparison of bacterial colony growth on the control and the treatment that grew on solid media was calculated according to the set contact time of 1, 2, and 5 minutes. The effectiveness of antiseptics is assessed by calculating according to the percentage kill principle. The results of the calculation of *C. albicans* colonies in contact time for 1, 2, and 5 minutes were 62.39%, 80.85%, and 90%, respectively. This finding shows that the contact time of 5 minutes has good effectiveness. Therefore *M. oleifera* leaves have the potential to be an effective antiseptic against *C. albicans.*
Keywords : Antiseptic; *Candida albicans*; *Moringa oleifera*; Percentage kill

INTRODUCTION

Candida albicans (*C. albicans*) is a commensal organism commonly found as a conjunctival, oral, genital, gastrointestinal tract, and other body parts (Spampinato et al. 2013). However, compromised immune conditions can lead to opportunistic infection with *C. albicans.* Opportunistic infections can only affect external tissues, such as mucous membranes or skin, until they spread throughout the body through blood vessels and into various organ (Rafiq, 2023).

The cell wall of *C. albicans* is made up of protein, chiline, and glucan. The cell wall of this fungus is tasked with protecting cells from environmental threats, such as temperature changes, dehydration, defense against the immune system, and osmotic changes. The cell membrane of *C. albicans* plays an important role as a cell communication pathway. The presence of sterols in the cell membrane plays an important role as a provider of rigidity, resistance, and stability of the cell against external stimuli. The most abundant type of sterols is ergosterol which is synthesized by the lipid body and endoplasmic reticulum. Ergosterol is a characteristic of fungal cell membranes (de Oliveira et al. 2013).

The polymorphism of *C. albicans* allows these organisms to change shape, from commensal to pathogenic. In the communicative state, *C. albicans* has a yeast form. Changing its shape into invasive hyphae will cause this organism to be able to invasively penetrate the tissue and trigger endocytosis. An important signaling pathway for hyphae formation is the cAMP-dependent protein kinase A. Adhesion is facilitated by ALS3 and Hwp1 proteins that recognize and bind host cell membrane ligands, such as fibrinogen, fibronectin, and proteins. The presence of hydrolytic enzymes, also aids in the attachment to the host cell and the destruction of the host cell. Cannabinoid toxin has an important role in the incidence of candidiasis which can trigger damage to host cells leading to systemic infections and death. In addition, the formation of biofilms also increases morbidity and mortality (de Oliveira et al. 2013).

Antiseptics are biocides that function to inhibit or kill microorganisms found in living tissues. Examples of antiseptics are surgical scrubs and hand soaps. Disinfectants are the same products as antiseptics, but they are commonly used for dead surfaces or objects. Antiseptics have

***Corresponding author : Conny Riana Tjampakasari**
Email : connyrianat@yahoo.com

a variety of main ingredients that have antimicrobial effects. Among them are alcohol, aldehydes, analyses, biguanides, diamidines, halogen-releasing agents, chlorine-releasing agents, silver compounds, peroxygen, phenols, bis-phenols, halophenols, quaternary ammonium compounds, and fume sterilizers. Various studies have been conducted to determine the mechanism of action of antiseptics, such as checking the absorption, leakage, and lysis of intracellular components; microscopic analysis of cells exposed to biocides; impact on the model membrane; changes in cell homeostasis; impact on macromolecular biosynthetic processes; interaction with macromolecules; and enzyme inhibition, electron transport, oxidative phosphorylation is some of them (Kanagalingan et al. 2015).

The use of antiseptics is one of the useful agents for the prevention and treatment of infections, including fungal infections, in the skin, teeth, and mouth area. Povidone iodine antiseptics, based on in vitro research, are known to have the ability to kill *C. albicans* in a span of 10-12 seconds. Antiseptics with the active content of octenidine dihydrochloride and phenoxyethanol have also been shown to be effective in treating vulvavaginal candidosis. However, there are some reports that the use of these synthetic antiseptics can cause irritation, burning sensation, itching, blisters and contact dermatitis (Friese et al, 2003)

Moringa oleifera (M. oleifera) is a plant native to Asia and Africa. This plant belongs to the Moringaceae family and has 13 species spread across tropical and subtropical climates, in Indonesia it is known as *Moringa*. The plant is famous for its flowers, fruits, and leaves that can be used for medicine, food, and cosmetic oils. This plant has a height of 5 to 10 meters (Lowe et al, 2006).

There are various bioactive components contained in this plant and beneficial for humans, including glycosides, phenolic acids, pterygospermine, steroids, saponins, flavoid, benzylglucosinoalte, vitamins, alkaloids, polyphenols, tannins, and carotenoids. The bioactive component content contained in M. oleifera leaves is widely used for pharmacological purposes (Vergara et al, 2017).

This plant has a variety of benefits, one of which is as an antimicrobial, including as an antifungal (Ayirezang et al. 2020). *M. oleifera* leaf extract has been shown to inhibit the growth of *S. epidermidis* during 24-hour incubation at 37°C by disc diffusion method. Other studies have also been conducted to determine the antimicrobial activity of *M. oleifera* by the same method, but using different bacteria, namely *S. aureus* and *E. coli.* The results showed that the antimicrobial leaf extract of this plant against both bacteria. (Jahan et al. 2020). Various studies have also shown the effectiveness of *M. oleifera* extract against the growth of *C. albicans. M. oleifera* seed extract (100%) has been shown to have moderate inhibitory power against the growth of *C. albicans* (Ervianingsih et al. 2019) Another study found that ethanol extract of *M. oleifera* leaves produced a larger inhibition zone than Nistatin, which is a commonly used drug for fungal infections (Bhattacharya et al. 2018). *M. oleifera* leaf extract was also proven to have antimicrobial activity by percentage kills test against *E. coli* and *S. pyogenes* bacteria (Sulistyani et al. 2023).

These contents are the ingredient in *M. oleifera* with antifungal effects. Until now, research on the potential of *M. oleifera* as an antiseptic against *C. albicans* has never been conducted. The various contents of *M. oleifera* leaves are expected to be useful antiseptics for the prevention of *C. albicans* infection. *M. oleifera* leaf extract is expected to be a natural antiseptic alternative that can be used as an antifungal agent to prevent *C. albicans* infection. Therefore, this study aims to determine the effectiveness of *M. oleifera* leaf extract as a natural antiseptic alternative by using a percentage of killing test with a specific contact time (Balouiri et al. 2016; Talapko et al. 2021)

MATERIALS AND METHODS

Extract preparation

The extract was obtained from the Department of Medical Pharmacy, Faculty of Medicine, University of Indonesia, weighing 120.8495 grams, 70% alcohol (Solvent, Indonesia) with a water concentration of 15.30% so that the purity of the extract was obtained 84.70%. *M. oleifera* ethanol extract is diluted by aqueous with 1% carboxymethylcellulose (Tradeindia, India) as solvent immobilization. The concentration used in this study was 800 mg/ml or equivalent to 80%.

Fungal preparation

The microbes used in this study are the fungus *C. albicans* ATCC 14053 (Multi Redjeki Kita, Indonesia) obtained from the Microbiology Laboratory, Department of Clinical Microbiology, Faculty Medicine, Universitas Indonesia. In 10 ml of Tripsoy Broth (Oxoid, UK) and 1 inoculation loop of test fungal was added which was previously equalized with Mc Farland 0.5 (BD PhoenixSpec, USA). The suspension will then be diluted until

dilution 10^5. The results of the dilution will be used as fungal examination (Babalska et al. 2021).

Antiseptic examination of the extract against *C. albicans* using the Percentage Kill Test

It consists of work on treatment (*M. oleifera* extract) and control carried out under the same conditions and time.

Treatment Groups

First, 0.5 ml of microorganism suspension was added to 4.5 ml of *M. oleifera* extract as a treatment. Then, homogenization with vortex is carried out. After 1 minute, using a micropipette (Gilson, USA), take 1 ml of solution that has been homogenized with vortex (Scientific indutries, USA) and mix with 9 ml of sterile aquatics. Homogenization is carried out again. After 2 minutes, using a pipette take 1 ml of solution that has been homogenized with vortex and mixed with 9 ml of sterile aquades (UKK LMK, Indonesia). Then the vortex returns. After 5 minutes, using a micropipette take 1 ml of the solution that has been homogenized with vortex and mix with 9 ml of sterile aquatics then homogenize. For each dilution result, each solution is taken 1 ml using a pipette and put into 3 sets of sterile petri dishes. Each petri dish is added 15 ml of Sabouraud Dextrose Agar (SDA) (Oxoid, UK) in a warm condition of 45°C. The petri dish is then shaken to the left 6 times and to the right 6 times so that the mixture is evenly distributed. After this, incubate for 18-24 hours at a temperature of 35°C-37°C (Memmert, German). Using an electronic colony counter (Indiamart, India), the number of colonies in each petri dish (Thermofisher, Indonesia) is calculated for each growing colony (Tjampakasari et al. 2022).

Control Group

The control group was carried out simultaneously with the treatment group. In the same way, the addition of 0.5 ml of suspension of microorganisms is mixed into into 4.5 ml of sterile aquades (UKK LMK, Indonesia). Then, homogenization with vortex is carried out. After 1 minute, using a micropipette (Gilson, USA), take 1 ml of solution that has been homogenized with vortex and mix with 9 ml of sterile aquatics (UKK LMK, Indonesia). Homogenization is carried out again. After 2 minutes, using a pipette take 1 ml of solution that has been homogenized with vortex and mixed with 9 ml of sterile aquades (UKK LMK, Indonesia). Then the vortex returns. After 5 minutes, using a micropipette take 1 ml of the solution that has been homogenized with vortex and mix with 9 ml of sterile aquatics then homogenize. For each dilution result, each solution is taken 1 ml using a pipette and put into 3 sets of sterile petri dishes. Each petri dish (Thermofisher, Indonesia) is added 15 ml of Sabouraud Dextrose Agar (SDA) (Oxoid, UK) in a warm condition of 45 °C. The petri dish (Thermofisher, Indonesia) is then shaken to the left 6 times and to the right 6 times so that the mixture is evenly distributed. After this, incubate for 18-24 hours at a temperature of 35 °C -37 °C (Memmert, German). Using an electronic colony counter (Indiamart, India), the number of colonies in each petri dish is calculated for each growing colony (Tjampakasari et al. 2022).

Quality control of experiments

Inoculation was also carried out on 3 medium of of Sabouraud Dextrose Agar (SDA) (Oxoid, UK) as experimental quality control. The first tube contains 15 ml of SDA in a warm condition of 45 °C, used as a control medium, the second tube contains 15 ml of SDA (Oxoid, UK) added 0.5 ml of *C. albicans* as a microbial control and the third tube contains 15 ml of SDA added 0.5 ml of extract as an extract control. The petri dish (Thermofisher, Indonesia) is then shaken to the left 6 times and to the right 6 times so that the mixture is evenly distributed. After this, incubate for 18-24 hours at a temperature of 35°C-37°C (Memmert, German). Using an electronic colony counter (Indiamart, India), the number of colonies in each petri dish (Thermofisher, Indonesia) is calculated for each growing colony (Tjampakasari et al. 2022).

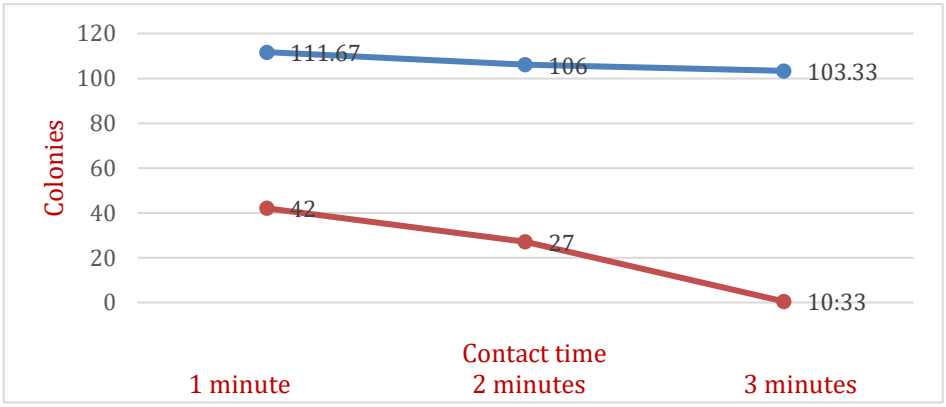
Percentage Kill Calculation

The Percentage Kill result is calculated by calculating the difference between the number of colonies growing in the control and the number of colonies in the extract, then dividing by the number of colonies in the control and multiplying it by 100%. This calculation is done for each contact time. The result is declared effective if the value obtained is ≥90**%** **(**Rutala et al. 2019; Rasyid et al. 2017).

RESULTS

This study assessed the growth of *C. albicans* colonies in the control and treatment given *M. oleifera* leaf extract with contact time of 1, 2, and 5 minutes using the percentage kill method with standard deviation which can be seen in Table I.

Quality control of experiments shows in the media control and the extract control, in SDA there is no growth, indicates that the media and extracts are in a sterile, uncontaminated state, while in the microbial control, SDA indicates the presence of fungal growth.



Blue line: control, Red line: extract

Figure I. Average Growth of *C. albicans* colonies on Controls and Extracts

Table I. Number of *C. albicans* colonies between Control and Extract

Contact Time	Number of colonies Control			Average colonies(C) ± SD	Number of colonies Extrak			Average colonies (E) ± SD
	I	II	III		I	II	III	
1 Minute	120	117	98	111.67 ± 11.71	50	41	35	42 ± 7.55
2 Minutes	112	107	99	106 ± 6.56	35	27	19	27 ± 8.00
5 Minutes	109	104	97	103.33 ± 6.00	12	10	9	10.33 ± 1.51

I, II, III: Number of repetitions, C: Control, E: Extract; SD: Standar Deviation

In the control, the growth results of *C. albicans* colonies with 3 repetitions in contact time for 1 minute were, 120, 117, and 98 colonies. These results showed a decrease in the number of colonies in contact time for 2 minutes of 112, 107, 99 colonies respectively with 3 repetitions. Meanwhile, the number of colonies that grew in contact time for 5 minutes in a row was 109, 104, and 97 colonies.

The number of *C. albicans* colonies given *M. oleifera* extract treatment with 3 repetitions in contact time for 1 minute in a row was 50, 41, and 35 colonies. The number of colonies shows a decrease in the contact time was 2 minutes for 35, 27, and 19 colonies respectively with 3 repetitions. As for the contact time of 5 minutes, the number of colonies that grow is 12, 10, and 9 colonies, respectively.

The average growth yield of *C. albicans* colonies in the control was 111.67, 106 and 103.33 for contact times of 1, 2 and 5 minutes, respectively. Meanwhile, the average growth yield of *C. albicans* colonies in the extract was 42, 27 and 10.33 for contact times of 1, 2 and 5 minutes, respectively (Figure I). The average results of the control and treatment have been set standard deviation (Table I).

The results of colony growth in the control and extract were then processed based on the percentage kill method formula (Table II).

Table II. Percentage Kill Test Results

Contact time	Percentage Kill Result (%)
1 Minute	62.39
2 Minutes	80.85
5 Minutes	90

Based on these results, it can be concluded that the longer the contact time, the better the antiseptic ability of *M. oleifera* extract to be able to radiate *C. albicans*. The percentage kill test has good results if the percentage produced is ≥ 90% for all three contact times. Although at 1 and 2 minutes of contact time, *M. oleifera* extract has not met a good percentage kill test, but at the time of contact for 5 minutes, it has achieved a 90% reduction in the number of *C. albicans* colonies.

DISCUSSION

This study shows how *M. oleifera* leaf extract can inhibit the growth of *C. albicans* colonies. This result can be seen from the comparison of the number of fungal colonies that grow in the control group and the treatment. It was found that the number of colonies in the treatment group had a considerable distance from the control group where the influence of *moringa* leaf extract worked in inhibiting fungal growth.

The results of this study were also equipped with control media, where the SDA used there is no

growth after the incubation period. The control of the extract, showed the same, there is no growth after the incubation period. A different thing is shown in the microbial control, after the incubation period SDA shows the presence of fungal growth. The results of this control test showed the suitability where the media and extracts were sterile and uncontaminated while the *C. albicans* used was in a live state.

The standard deviation values in the control and treatment for each contact time showed good results where in general the standard deviation obtained was smaller than the average value. A standard deviation lower than the mean value in the group indicates that the data tend to be close to the mean value, meaning that it has a low variance. Based on this, it can be stated that the data obtained both in control and treatment show consistency.

The results of the study show that the longer the contact time between the extract and *C. albicans*, the higher the killing power, this can be seen in the calculation of the percentage kill test where in the first minute the results obtained were only 62.39%, then in the second and fifth minutes the percentage kill test results were 80.85% and 90% which means that the longer the extract is in contact with microorganisms, the more inhibited the growth of fungal will be.

The maximum contact time required to determine the potential extract according to the percentage kill terminology is 5 minutes. As well as antiseptics, they are used to inhibit microbes on our skin with a relatively short use time.

C. albicans has a cell wall as the outermost protective structure. The most important components of the cell wall of *C. albicans* are chitin, glucans and mannoproteins. Chitin has mechanically strong properties and is insoluble in water. Thus, chitin can form a strong structure to protect fungal cells. Glucans are complex polysaccharides that are glucose polymers, rigid in nature, serving to provide structural strength to the fungal cell wall. Meanwhile, mannoproteins are essential in morphogenesis and pathogenesis because they function as environmental sensors. These factors make this fungus difficult to eradicate (Yusran et al 2020; Abbas et al. 2021).

Research conducted by Sulistyani H et al at Gadjah Mada University (2023) showed that there was an inhibition of the growth of *C. albicans* in *M. oleifera* leaf extract with dimethylsulfoxide solvent with graded concentrations (0.78%, 1.57%, 3.13%, 6.25%, 12.5%, and 25%). The study showed a minimum inhibition concentration of 6.25%. Thus, this study is continuous to determine the efficacy of *M. oleifera* leaf extract as an antiseptic against *C. albicans*.

This study is the first study to test the ability of *M. oleifera* leaf extract to kill *C. albicans* colonies with a percentage kill test. This study has limitations, namely only using extracts with one concentration and control using aqueducts without the addition of 1% carboxymethylcellulose. Based on the results of this study, *M. oleifera* extract is proven to have the potential as an antiseptic that can inhibit growth and kill *C. albicans* colonies. There was a colony growth inhibition that was seen from a decrease in the number of colonies in the extract compared to the number of colonies in the control at all contact times. The contact time that is considered effective is 5 minutes because it results in a kill percentage of 90%. This shows the good effectiveness of *M. oleifera* as an antiseptic against *C. albicans.*

The ability of *M. oleifera* extract as an antimicrobial is thought to be due to the various bioactive contained in it. The antifungal effect of tannins is caused by disturbances in the formation of fungal cell walls that lead to the death of organisms. In addition, the phenol content in these plant extracts also interferes with the integrity of fungal cell membranes and spore germination (McDonnell et al. 1999). The effects of spore germination disorders are caused by enzyme denaturation that causes disturbances in amino acids that are beneficial for germination (Teska et al. 2019).

The content of flavonoids can result in permeability disorders in the fungal cell membrane due to protein denaturation which causes inhibition of fungal growth. Flavonoids can also form compounds combined with phenol and ergosterol to make the pores in fungal cells larger which can make proteins and nucleic acids of the cell come out resulting in cell death (Kalpana et al. 2013).

The alkaloid content can prevent DNA replication through insertion into the DNA causing growth disorders. In addition, tannin content also inhibits fungal growth through impaired cell wall synthesis due to impaired chitin synthesis. Saponin compounds play a role in impaired cell membrane stability leading to lysis of fungal cells resulting in death.14 It is important to note that the inhibition effect of fungal growth may not be caused by a single bioactive component, but by the synergistic effect produced by the various bioactive components contained in the extract (Acsa et al. 2021).

Research by Tjampakasari et al (2022) also conducted a percentage kill test study with

M. oleifera extract but against different microorganisms, namely *E. coli* and *Streptococcus pyogenes*. Test results on *E. coli* at minutes 1, 2 and 5 showed results of 93.41%, 94.14%, and 96.87%, respectively, while against *S. pyogenes* 73.27%, 83.15% and 94.19%, respectively. *M. oleifera* effectively eliminates *E. coli* because the percentage kill value for all contact times was ≥90% whereas for *S. pyogenes*, *M oleifera* leaf extract showed good microbial activity at 5 minutes of contact time (94.19%).

The results of the study showed that Gram-positive microorganisms (*S. pyogenes*) were more difficult to inhibit by *M. oleifera* than Gram-negative microorganisms (*E. coli*). This result is in accordance with our study which showed that Gram-positive *C. albicans* gave an effective result only at 5 minutes (90%).

In addition, the study conducted by Kalpana et al (2013) showed similar results to our study. The research conducted using the modified Kirby-Bauer method showed that the average inhibition zone formed in *Streptococcus pneumonia* bacteria (Gram positive) given *M. oleifera* leaf extract had a smaller size compared to *E. coli* and *Klebsiella pneumonia* (Gram negative).

Meanwhile, the results of a study conducted by Acsa et al (2021) show different results. Based on the results of his research, Gram-positive bacteria are generally more sensitive to chemicals than Gram-negative bacteria. This is because the peptidoglycan layer as the outermost wall of Gram-positive bacteria is easily accessible by antimicrobial agents, while Gram-negative bacteria have a lipopolysaccharide layer as the main component of the outermost membrane of these bacteria.

LPS creates a permeability barrier on the cell surface, most Gram-negative bacteria exhibit intrinsic resistance to various antimicrobial treatments. Gram-negative bacteria use a complex mechanism to modify the Lipid A portion, which is the most bioactive component, of lipopolysaccharides (LPS) to adapt to their host environment (Nweke et al. 2015; Ahmadu et al.2021). *Candida albicans* whose cell wall resembles Gram-positive, so that it has the same ability as Gram-positive bacteria in terms of being inhibited by chemical effects (Farhana A et al. 2023).

As a Gram-positive microorganism, *Candida albicans* is so difficult to eradicate but with *M. oleifera* it has proven to have the potential to be used as an alternative antiseptic because of its ability to kill the fungus within 5 minutes. In the future, *M. oleifera* has great hopes to be used as an antiseptic alternative not only against *C. albicans* but also for other microbes.

CONCLUSION

The percentage kill values obtained in this experiment were 62.39%, 80.85%, and 90% for contact time of 1, 2, and 5 minutes. *M. oleifera* leaf ethanol extract with a contact time of 5 minutes is effective as an antiseptic. *M. oleifera* leaves have the potential to be an effective antiseptic against *C. albicans* and the extract has hope to be used as an herb-based antiseptic alternative.

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CONFLICT OF INTEREST

There is no conflict of interest in the writing of this study

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