Original Research Report

ANTIFUNGAL ACTIVITY OF *Selaginella plana* (Desv. ex Poir.) Hieron EXTRACT AGAINST *Candida albicans* IN VITRO

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ABSTRACT

Candidiasis is an opportunistic infection caused by *Candida albicans*. This infection commonly affects the skin, oral mucosa, vagina, and gastrointestinal tract. Excessive use of azole antifungals for treating *Candida albicans* infections can lead to the development of resistance. Therefore, it is necessary to explore alternative treatments using medicinal plants such as *Selaginella plana*, commonly referred to as "*rutu-rutu*" in a local language spoken across Maluku, Indonesia. *Selaginella plana* contains active compounds from various chemical classes, including terpenoids, steroids, flavonoids, and saponins. This study aimed to determine the ability of *Selaginella plana* extract as an antifungal agent against *Candida albicans* by evaluating its inhibitory and antifungal effects. This study used an actual experimental design and broth dilution method. The research methodology involved the extraction of *Selaginella plana* using a solvent of 96% ethanol. The extract was then prepared in various concentrations, i.e., 100%, 50%, 25%, 12.5%, 6.25%, and 3.125%. Additionally, ketoconazole and distilled water were included in the experiment for the positive and negative controls, respectively. The results of this study demonstrated that *Selaginella plana* extract inhibited the growth of *Candida albicans* when administered starting at a concentration of 12.5%. However, the antifungal potential of *Selaginella plana* extract that induced cell death was only observed at a concentration of 100%. The fungicidal activity was exclusively identified in the undiluted, pure extract. The inhibitory and cytotoxic effects of *Selaginella plana* on *Candida albicans* cells were attributed to the presence of bioactive compounds in *Selaginella plana*, including flavonoids, tannins, terpenoids, and saponins. These bioactive compounds had the ability to inhibit cell growth by altering membrane permeability, causing mitochondrial dysfunction, and disrupting ergosterol biosynthesis. It can be concluded that *Selaginella plana* extract can act as a fungistatic agent against the proliferation of *Candida albicans*.

Keywords: Antifungal medicine; *Selaginella plana*; *Candida albicans*; infectious disease; medicine

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Article history
●Submitted 15/3/2023 ● Revised 15/7/2023 ● Accepted 21/8/2023 ● Published 10/9/2023

How to cite: Warella JC, Rahma K, Widodo ADW, et al (2023). Antifungal Activity of *Selaginella plana* (Desv. ex Poir.) Hieron Extract Against *Candida albicans* In Vitro. Folia Medica Indonesiana 59 (3), 295-301, https://doi.org/10.20473/fmi.v59i3.44165

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pISSN:2355-8393, eISSN: 2599-056x

Highlights:
1. A study on the medical benefits of *Selaginella plana* has significant academic value due to its extensive traditional usage among the Moluccan people as a medicinal remedy, especially for its antifungal properties.
2. The findings of this study will allow further screening to determine the mechanism of action of bioactive compounds in inhibiting the growth of *Candida albicans*.

INTRODUCTION

Fungal infections pose microbial threats to human health. According to a study conducted by Bongomin et al. (2017), fungal infections cause about 1.5 million deaths each year. Mortality rates caused by fungal infections continue to increase despite improvements in social development and health status. *Candida albicans* is the most common fungus responsible for human infections. *Candida albicans* is a normal flora commonly found in the skin, vagina, digestive tract, and respiratory tract. However, it also has the potential to induce opportunistic infections. *Candida albicans* can

invade tissues and organs, affecting about 50% of the world's population (Naglik et al. 2014). One of the surgical infections caused by *Candida albicans* is candidiasis, which can infect the surface of the skin, oral mucosa, and penis. The predominant cause of invasive candidiasis was *Candida albicans*, accounting for 46.3% of cases, followed by *Candida glabrata* (24.4%) and *Candida parapsilosis* (8.1%) (Andes et al. 2016). Candidiasis is commonly referred to as a yeast infection because the causative agent is yeast. Candidiasis frequently manifests as a secondary infection in individuals with weak immune systems (Qadir & Asif 2020).

The global trend in candidiasis prevalence is concerning, as *Candida* species continue to be the main causative agents of invasive fungal infections. On a global scale, the annual prevalence of invasive candidiasis exceeds 750,000 cases (Bongomin et al. 2017). Prevalence data from a study by Pegorie et al. (2017) showed that there were 5,142 candidiasis cases in England. The data also revealed 28,991 cases in Brazil, 38,795 cases in Pakistan, 11,840 cases in Russia, and 4,540 cases in Vietnam. The Mycology Division of the Department of Dermatology and Venereology, Dr. Soetomo General Academic Hospital, Surabaya, Indonesia, reported candidiasis cases in the period of 2013–2016. According to the report, the prevalence rates of candidiasis were observed to be 6.23% (99 patients) in 2013, 6.08% (77 patients) in 2014, 5.58% (55 patients) in 2015, and 8.97% (67 patients) in 2016 (Puspitasari et al. 2019).

The main classes of antifungal drugs used for the treatment of *Candida* infections include azoles, polyenes, echinocandins, pyrimidines, and allylamines. Although these antifungal drugs have been widely used to treat *Candida albicans* infections, misuse and overuse of these treatments can result in the development of resistance. According to data from the Centers for Disease Control and Prevention (2019), there has been a significant increase in *Candida albicans* resistance to antibiotics, with a total of 34,800 recorded infection cases and 1,700 deaths. This indicates that the efficacy of antifungal drugs in treating infections caused by *Candida albicans* has been limited, which leads people to seek alternative treatments using medicinal plants. Plants produce a variety of secondary metabolites that function as defense compounds against herbivores and microorganisms (Wink 2015). *Selaginella plana* is a medicinal plant commonly used by the Moluccan people in Indonesia. The plant is referred to as "*rutu-rutu*" in a native language spoken in the Maluku region of Indonesia.

Selaginella plana has been used as an alternative medicine in several traditional medicinal practices, including wound healing, postpartum care, management of menstrual disorders, treatment of skin diseases, headache relief, as well as treatment for fever, respiratory tract infections, urethral infections, cirrhosis, cancer, and rheumatism. *Selaginella plana* is known to have a diverse range of bioactivities, including antitumor, antibiotic, antiviral, antioxidant, hypoglycemic, anti-inflammatory, antimalarial, antidiabetic, and anti-aging effects (Xu et al. 2019). In our previous study, we examined the beneficial effects of *Selaginella plana* as an antimicrobial agent capable of inhibiting the growth of *Staphylococcus aureus*. People use *Selaginella plana* as a traditional medicine because it contains secondary metabolites such as alkaloids, flavonoids, and terpenoids (Warella et al. 2020). However, the use of *Selaginella plana* as an antifungal agent has never been reported. Hence, this study aimed to test the potential of *Selaginella plana* extract as an antifungal agent in inhibiting the growth of *Candida albicans*.

MATERIALS AND METHODS

This study was a pure experiment using a randomized post-test-only control group design. The experiment consisted of three repetitions conducted on six experimental groups, along with a positive control and a negative control. An observation of the experimental groups was performed in relation to the application of six treatment concentrations (Parnomo 2021). The fungal specimens were pure cultures of *Candida albicans* ATCC 10231 obtained from the Surabaya Health Laboratory Center, Surabaya, Indonesia (7°16'2.53328"S, 112°45'37.13756"E). The experimental samples included different concentrations of *Selaginella plana* (Desv. ex Poir.) Hieron extract sourced from Kairatu Village, Maluku, Indonesia (3°12′55″S, 128°10′34″E). The identification of *Selaginella plana* plants was conducted at the Plant Conservation Center, located within Purwodadi Botanical Garden in Pasuruan, Indonesia. The objective of this procedure was to match the observed characteristics of the *Selaginella plana* samples with the plant identification key, which follows the binomial nomenclature classification system. This procedure was carried out as it served the purpose of identifying the specific plant species before conducting the experiment. It was helpful in minimizing errors in the collection of samples and mitigating the risk of unexpected contamination with other plant species.

The *Selaginella plana* extraction process was carried out using a maceration method for three 24-hour intervals. The extraction process incorporated 96% ethanol as a solvent. One notable advantage of the maceration method was that it was performed

without heating, allowing thermolabile compounds to be extracted. A total of 5 kg of *Selaginella plana* leaves were washed, cut into small pieces, dried in an oven at 40–60°C, and pulverized into powder. After that, 100 g of powder were added to 1 L of 96% ethanol. The mixture was then left to stand for one night with occasional stirring. The next step was separating the ethanol from the *Selaginella plana* extract using an evaporation flask at 60°C (Septiani et al. 2021).

The fungal rejuvenation process was carried out by obtaining a smear of *Candida albicans* from a pure culture using an inoculation loop. Afterwards, the samples underwent incubation for 72 hours at a temperature of 37°C on Sabouraud dextrose agar. The purpose was to sustain the viability and purity of the organisms, prevent any variation or mutation, and generate a new and fresh culture for successful reproduction (Saigal et al. 2011). *Candida albicans* suspensions were prepared in test tubes using 10 mL of Mueller-Hinton broth medium. The researchers put a smear of the rejuvenated microbial samples using an inoculation loop into the Mueller-Hinton broth medium. Following that, we carried out the vortex process until they achieved homogenous suspensions.

The experiments were conducted using the dilution method, following the guidelines set by the Clinical and Laboratory Standards Institute (2020), to determine the minimum inhibitory concentration and minimum fungicidal concentration (MFC). The application of the dilution method offered an advantage in assessing the efficacy of the compounds in terms of inhibitory and fungicidal properties against *Candida albicans*. In addition, this method enabled the quantitative assessment of microorganisms and facilitated the simultaneous evaluation of multiple concentrations within a single experimental assay. The Mueller-Hinton broth was used as the medium in this study. In this experiment, there were six treatment groups with different extract concentrations, i.e., 100% (P1), 50% (P2), 25% (P3), 12.5% (P4), 6.25% (P5), and 3.125% (P5). Additionally, this experiment included a positive control (K+) and a negative control (K-) that received ketoconazole and distilled water, respectively.

The first step was to obtain a bacterial suspension equivalent to the 0.5 McFarland standard. In each of the treatment groups (P1, P2, P3, P4, P5, and P6), 1 mL of *Selaginella plana* extract at predetermined concentrations was combined with 1 mL of Mueller-Hinton broth. The mixture was put into a vortex mixer to allow homogenization of the solution within the test tubes. Afterwards, the treatment mixture was added to 1 mL of microbial suspension for each of the treatment groups. The procedure was replicated three times. Following a 72-hour incubation period at a temperature of 37°C within an incubator, the treatment groups were observed and compared with both the positive and negative control groups (Fitriana et al. 2020).

The minimum inhibitory concentration was assessed for both the treatment groups and control groups following a 72-hour incubation period. The determination of the minimum inhibitory concentration was conducted by observing small samples and identifying which concentration of the *Selaginella plana* extract could effectively block fungal growth, as visually confirmed by three observers. The visual observations required the identification of any turbidity within the experimental groups. The presence of turbidity indicated that a fungal growth was observed to be positive. In contrast, the absence of turbidity indicated the absence of fungal growth. Subsequently, the minimum inhibitory concentration was established (Kowalska-Krochmal & Dudek-Wicher 2021). In addition, the establishment of the minimal fungicidal concentration involved the observation of a culture on Mueller-Hinton agar, wherein the concentrations of the extract varied. This observation was conducted over a period of 72 hours at a constant temperature of 37°C. The *Candida albicans* colonies were measured using a colony counter. The *Selaginella plana* extract was determined to have fungicidal properties when the number of colonies was below 10. However, its efficacy in killing *Candida albicans* diminished when the colony count exceeded 10 colonies (Balouiri et al. 2016).

RESULTS

This study analyzed the administration of *Selaginella plana* extract using the broth dilution method. In this experimental approach, the researchers employed a sequential dilution process to produce a range of concentrations from the extract. The concentrations obtained were 100%, 50%, 25%, 12.5%, 6.25%, and 3.125%. Distilled water served as the negative control, whereas ketoconazole was used as the positive control. Table 1 shows the results of the minimum inhibitory concentration test conducted on the *Selaginella plana* extract.

The experimental findings indicated that the extract derived from *Selaginella plana* had inhibitory effects on *Candida albicans*, with inhibition observed at concentrations of 100% up to 25%. Consequently, a concentration of 25% was determined to be the minimum inhibitory concentration. The group that received ketoconazole as a positive control did not exhibit any turbidity, but

the control group that received distilled water as a negative control displayed turbidity.

Table 1. Minimum inhibitory concentration test results of *Selaginella plana* extract against *Candida albicans*.

Groups	Fungal growth		
	1st replicate	2nd replicate	3rd replicate
100%	-	-	-
50%	-	-	-
25%	-	+	-
12.5%	+	+	+
6.25%	+	+	+
3.125%	+	+	+
Positive control	-	-	-
Negative control	+	+	+

Notes:
(-): Absence of fungal growth.
(+): Presence of fungal growth.

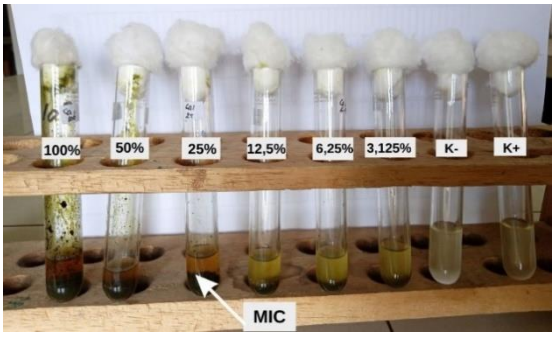

Figure 1. Observation of the minimum inhibitory concentration of *Selaginella plana* extract.

A test was undertaken to determine the minimum inhibitory concentration (MIC) of *Selaginella plana* extract at various concentrations (100%, 50%, 25%, 12.5%, 6.25%, and 3.125%). The experiment involved a comparison with ketoconazole and distilled water as the positive and negative controls, respectively. Figure 1 shows that the minimum inhibitory concentration of *Selaginella plana* extract was determined to be 25%, as indicated by the white arrow.

Table 2 presents the results of the examination on the minimum inhibitory concentration of *Selaginella plana* extract against *Candida albicans*. The results from the experiment indicated that the *Selaginella plana* extract had the ability to inhibit the growth of *Candida albicans* when administered at a concentration of 100%. Figure 2 depicts the proliferation of *Candida albicans* colonies on the petri dishes. Only a single colony of *Candida albicans* was observed within the area where a 100% concentration of *Selaginella plana* was administered. Hence, the presence of less than 10 colonies of *Candida albicans* was interpreted as a negative result.

Tabel 2. Minimum fungicidal concentration test results of *Selaginella plana* extract against *Candida albicans*.

Groups	Minimum fungicidal concentration		
	1st replicate	2nd replicate	3rd replicate
100%	-	-	-
50%	+	+	+
25%	+	+	+
12.5%	+	+	+
6.25%	+	+	+
3.125%	+	+	+
Positive control	-	-	-
Negative control	+	+	+

Notes:
(-): Absence of fungal growth.
(+): Presence of fungal growth.

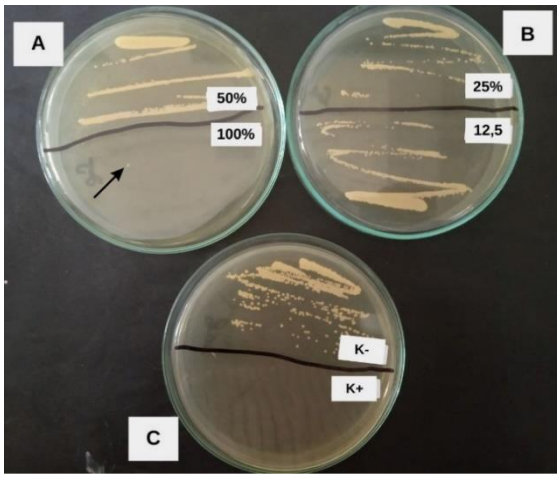

Figure 2. Minimum fungicidal concentration test of *Selaginella plana* extract at concentrations of 50% and 100% (A), 25% and 12.5% (B), and ketoconazole and distilled water (C).

DISCUSSION

Candida albicans is a pathogenic microorganism that frequently causes infections in humans. Many studies have reported the emergence of antifungal medication resistance in *Candida albicans* (Ksiezopolska & Gabaldón 2018). The resistance has been observed to arise as a consequence of the extensive use of antifungal drugs, such as fluconazole and voriconazole, for both prophylactic

and therapeutic purposes. This situation becomes a potential background for the advancement of antifungal drugs derived from natural ingredients, which is presently an expanding industry (Augostine & Avery 2022). Compared to antifungal drugs made from synthetic chemicals, those made from natural ingredients have many advantages and lower toxicity levels.

In this study, the minimum inhibitory concentration was determined using the liquid microdilution method. According to a study conducted by Balouiri et al. (2016), this particular method is widely regarded as the most appropriate approach due to its ability to estimate the necessary concentration of antimicrobial agents. The findings of this study showed that *Selaginella plana* extract at specific concentrations (100%, 50%, and 25%) can inhibit the growth of *Candida albicans*. Consequently, it can be classified as a fungistatic agent. However, *Selaginella plana* extract at low concentrations cannot inhibit *Candida albicans*. The fungistatic ability of *Selaginella plana* is attributed to the presence of an active compound that inhibits the growth of *Candida albicans*, with the inhibitory effect becoming more evident as the concentration of the plant extract increases. All active compounds in Selaginella show high potential as effective antimicrobials against *Candida albicans* (Cao et al. 2015).

In a previous study, Janeczko et al. (2022) examined the antifungal activity of quercetin, a flavonoid compound, against *Candida albicans*. The study revealed a range of minimum inhibitory concentrations between 2 mg/mL and 256 mg/mL. The activity of quercetin causes inhibition of hyphae formation, enhances membrane permeability, and induces cell leakage in *Candida albicans*. The plasma membrane serves as a protective barrier that plays a crucial role in preserving microbial cell viability. However, the active compound can disrupt adenosine triphosphate (ATP) synthesis, leading to obstruction of extracellular transportation across the membrane. In addition, it is widely known that saponin compounds can destabilize the integrity of cellular membranes, thereby inducing osmotic stress that leads to the release of cell organelles and subsequent cell lysis (Nuraeni et al. 2020).

In the minimum fungicidal concentration test conducted in this study, *Selaginella plana* extract showed an effect against *Candida albicans* at 100% concentration. This result was proven by the lack of colonies that proliferated on the agar media. This might be because *Selaginella plana* contains secondary metabolites such as flavonoids, tannins, and saponins (Miftahudin et al. 2015). Other studies have focused on the effects of flavonoid compounds on *Candida albicans*. These studies found that amentoflavone increased reactive oxygen species (ROS) while also impeding the ergosterol biosynthesis of *Candida* fungi (Nascimento et al. 2018). Consequently, this disruption leads to mitochondrial dysfunction and cell death. Additionally, flavonoids (e.g., isoquercitrin and flavonol) can cause changes in cell membrane permeability, thus inhibiting cell growth (Nguyen et al. 2021).

In a previous study conducted by Shariati et al. (2022), it was shown that terpenoids have the capability to destroy the cell membrane structure of *Candida albicans*. These compounds can block the respiratory chain by inhibiting succinate dehydrogenase, an enzyme attached to the inner mitochondrial membrane of *Candida albicans*. Furthermore, it was determined that the *Selaginella plana* extract at concentrations ranging from 50% to 3.125% has no fungicidal effects against *Candida albicans*. Resistance to antifungal drugs can be attributed to various factors, including point mutations, increased expression levels of the lanosterol 14-α-demethylase (ERG11) gene, and enzymatic changes within the ergosterol biosynthetic pathway (Alizadeh et al. 2017). The 14α-demethylase (Erg11p) gene is involved in the biosynthesis of ergosterol, which serves as the primary sterol in the fungal cell membrane. In addition, the ability of *Candida albicans* to form biofilms is an essential factor in the development of resistance to antifungal drugs (Alikhani et al. 2022).

Strength and limitations

The strength of this study is the novel application of *Selaginella plana* extract as an antifungal agent specifically targeting *Candida albicans*, which has not been reported in previous research. Therefore, this study represents the first report of the inhibitory and fungicidal properties of *Selaginella plana* extract against the growth of *Candida albicans*, as demonstrated by the minimum inhibitory concentration and minimum fungicidal concentration test results. In addition, to determine the antifungal efficacy of *Selaginella plana*, the researchers used the agar dilution method to observe differences in the inhibitory and fungicidal power of the extract at different concentrations. The limitation of this study is that the researchers have yet to explicitly examine the ability of *Selaginella plana* bioactive compounds by observing the absorbance using spectrophotometry. Therefore, the measurement of absorbance is necessary to accurately determine the turbidity of the fungal suspension.

CONCLUSION

Selaginella plana extract possesses inhibitory properties against the growth of *Candida albicans*. At the same time, its fungicidal ability is effective only in the form of undiluted, pure extracts. Further research is necessary to explore the underlying mechanism of the active compounds present in *Selaginella plana* extract with regards to their efficacy as antifungal agents.

Acknowledgment

The authors would like to thank the staff of the Microbiology Laboratory of the Department of Medical Microbiology, Faculty of Medicine, Universitas Airlangga, Surabaya, Indonesia, for their assistance during the conduct of this research.

Conflict of interest

None.

Ethical consideration

The ethical approval for this research was issued by the Health Research Ethics Committee, Faculty of Dental Medicine, Universitas Airlangga, Surabaya, Indonesia, with certificate No. 766/HRECC. FODM/XII/2019 on 17/12/2019.

Funding disclosure

None.

Author contribution

All authors contributed to the conception and design of the study, analysis and interpretation of the data, drafting of the article, critical revision of the article for important intellectual content, final approval of the article, provision of the study materials, and collection and assembly of the data.

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