



In Vitro Antifungal Activity of Ethanolic Stem Extract of *Tinospora crispa* Against *Candida albicans* and *Malassezia furfur*

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Abstract

Background: Fungal infections such as candidiasis caused by *Candida albicans* and pityriasis versicolor caused by *Malassezia furfur* are highly prevalent in Indonesia. Conventional antifungal treatments often face challenges like resistance and adverse side effects. As an alternative, brotowali stem (*Tinospora crispa*) extract, rich in alkaloids, flavonoids, and tannins, shows promise as an antifungal agent. This research investigates the in vitro antifungal efficacy of ethanol extract from brotowali stem against *C. albicans* and *M. furfur*. **Methods:** A laboratory experimental design utilizing the well-diffusion method was applied, measuring inhibition zone diameters on Sabouraud Dextrose Agar after 2x24 hours at extract concentrations of 45%, 60%, 75%, and 90%. **Results:** The ethanol extract of brotowali stem exhibited statistically significant but relatively low antifungal activity based on inhibition zone diameters against *C. albicans* (0,002) and *M. furfur* (0,001), with higher concentrations showing greater antifungal. The largest inhibition zones measured 5.93 mm for *C. albicans* and 3.97 mm for *M. furfur*. **Conclusions:** The extract concentrations of 90% concentration exhibited moderate antifungal activity against *C. albicans*. For *M. furfur*, all concentrations demonstrated weak antifungal activity.

Keywords: Antifungal; *Candida albicans*; *Malassezia furfur*; *Tinospora crispa*

Introduction

Fungal infections are a serious health challenge for tropical countries such as like Indonesia. High temperatures and humidity support the growth of fungi that can infect humans. Every year, around 7.7 million Indonesians suffer from fungal infections, with candidiasis and dermatomycosis being the most prevalent. (Wahyuningsih et al., 2021).

The prevalence of candidiasis in Indonesia is estimated to range from 20–25% (Puspitasari et al., 2019), while data from Centers for Disease Control and Prevention 2019 recorded 34,800 cases of global candidiasis in 2017 (CDC, 2019). *Candida albicans* (*C. albicans*) is the main causative agent of candidiasis, with an average contribution of 56% of reported cases (Rodiah et al., 2022). This fungus can invade the skin, nails, hair, mucous membranes, vagina, mouth, and also the esophagus by transmitting through direct contact or via contaminated intermediary objects (Puspitasari et al., 2019).

Dermatomycosis is a fungal infection affecting the skin, nails, and hair, caused by dermatophyte fungi (Sofyan & Buchair, 2022). The population of dermatomycosis



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worldwide has increased significantly, reaching about 20-25% (Wakano et al., 2022). Pityriasis versicolor is known to be the second most common dermatomycosis disease in Indonesia (Anggraeni et al., 2021). This disease is caused by *Malassezia furfur* (*M. furfur*) by invading the stratum corneum of the skin (Tarigan & Graharti, 2022). It is estimated that approximately 140 million cases of *M. furfur* occur annually (Sunnah et al., 2020).

The high incidence of candidiasis and pityriasis versicolor emphasize the urgency of antifungal management in patients (Sunnah et al., 2020). Conventional antifungal therapy still faces limitations such as serious side effects, including impaired liver function, anaphylactic reactions, nausea, vomiting, and headaches (Herkamela & Yenny, 2022). Long-term use of azole group antifungal also increases the risk of resistance (Pristov & Ghannoum, 2019). Therefore, further research is needed to develop therapeutic alternatives from natural sources, which are considered to have fewer adverse effects.

With its abundant natural resources, Indonesia has great potential in the use of medicinal plants as alternative antifungal therapies, such as brotowali (*Tinospora crispa*). Brotowali is widely recognized as a traditional medicinal plant across Asia and Africa. This plant grows wild or vines in the yard of the household. Brotowali extract has many pharmacological properties, including anti-inflammatory, antioxidant, immunomodulatory, cytotoxic, antimalarial, cardioprotective cardio, and antidiabetic (Rahman et al., 2020).

Previous studies have demonstrated that ethanol extract of brotowali stem with a concentration of 75% effectively inhibits *Trichophyton rubrum* with a mean inhibition zone of 17.96 mm (Erza et al., 2020), and has strong antifungal activity against *Pityrosporum ovale* with 69.12% inhibition in shampoo formula (Nuryanti et al., 2015). This extract has also been shown to inhibit *C. albicans* biofilm formation at an optimal concentration of 1000 µg/ml (Hutomo et al., 2022). However, the antifungal activity of brotowali stem ethanol extract against *Candida tropicalis* has been reported to be weak (Hutomo et al., 2023). The presence of active compounds such as phenols, terpenoids, alkaloids, saponins, tannins, and flavonoids is believed to contribute to the antifungal properties (Erza et al., 2020; Hutomo et al., 2022). Given the urgency to find therapeutic alternatives from medicinal plants that have potential as antifungals, and the limited research on the antifungal activity of brotowali stems, this study aims to evaluate the in vitro antifungal efficacy of brotowali stem ethanol extract (*T. crispa*) against *C. albicans* and *Malassezia furfur*.

Method

This study employed an in vitro experimental laboratory design using the agar well diffusion method. The extract used was a 96% ethanol extract of brotowali (*T. crispa*) stems at concentrations of 45%, 60%, 75%, and 90%. These concentrations were prepared through volume/volume (v/v) dilution of the crude extract and were intended to evaluate relative antifungal activity across different concentration levels, rather than to determine the minimum inhibitory concentration (MIC). In this study design, the experimental subjects were divided into two groups, the treatment group given the extract and the control group. The control group consisted of a positive control treated with ketoconazole and a negative control treated with distilled water.

Preparation of Extract

The preparation of brotowali (*T. crispa*) stem extract was conducted at the Balai Penelitian Tanaman Rempah dan Obat (BALITTRO) using a cold extraction method, namely maceration. Brotowali stems were collected from a 3-year-old plants that grown at the BALITTRO Experimental Garden in Bogor, Indonesia, located at an altitude of 700 meters above sea level, with an annual rainfall of 3,974 mm in 2023 (BMKG, 2024). The stems selected were mature or hard ones. Harvesting was performed between 08:00—

10:00 a.m., followed by cleaning, cutting, and sun-drying under direct sunlight for three days between 08:00—12:00 a.m. Once dry, the stems were ground using a grinder to obtain a powdered simplicia. A total of 1,500 g of simplicia powder was then mixed with 96% ethanol and subjected to maceration for 1–2 days, filtered to separate the filtrate from the residue, and evaporated using a Rotary Evaporator for 6 hours at temperature of 40-50°C. The final product yielded 327 g of viscous brotowali stem extract, corresponding to an extraction yield of 23.8%. The concentrated extract was then carried out a phytochemical screening to identify the presence of chemical constituents or secondary metabolites in the brotowali stem, with the results presented in Table 1.

Table 1. Phytochemical Screening of Brotowali Stem

Chemical Compounds	Reagents	Results	Observations
Alkaloids	Dragendoff	+++	Formation of a brick-red to orange-colored solution
	Mayer	++	Formation of a whitish-yellow precipitate
	Wagner	+++	No brown coloration observed
Flavonoids	Mg + HCl	+++++	Formation of a brick-red to orange-yellow precipitate
Phenols	Distilled water + NaCl 10% + FeCl ₃ 1%	++++	Formation of dark blue to blackish-blue precipitate
Terpenoids (Steroids)	Distilled water + Ether + Lieberman-Burchad	++++	Formation of blue, bluish-green, dark green, and blackish-green solution
Tannins	FeCl ₃ 1%	+++++	Formation of dark green to blackish-green solution
Saponins	Distilled water+HCl ₂ N	+	Weak and unstable foam formation

Information: (+) Positive = Presence of the chemical compound

Antifungal Assay

Brotowali stem extract was diluted by the addition of sterile distilled water to obtain concentrations of 45%, 60%, 75%, and 90%. The positive control solution was prepared by dissolving 0.2 g of 2% ketoconazole in 10 mL of sterile distilled water. The negative control consisted of 10 mL of sterile distilled water. The sample size was determined using Federer’s formula to identify the minimum number of repetition for each treatment group (Thadeus et al., 2024), resulting in a minimum of four repetitions for each group.

Fungal suspensions were prepared by aseptically collecting cultures of *C. albicans* and *M. furfur* fungi using sterile ose. Each fungal culture was then suspended in 10 mL of 0.9% NaCl solution in separate test tubes. The fungal suspensions were adjusted to match the 0.5 McFarland standard.

In the preparation of Sabouraud Dextrose Agar (SDA), a double-layer method consisting of a base layer and a seed layer was employed. Base layer as the bottom layer was prepared by pouring 10 mL of SDA into a Petri dish. Then, after base layer hardened, a well plate was placed on top of it and a layer was added seed layer between the plates. The seed layer consisted of a mixture of SDA and fungal suspension at a ratio of 9:1. Once the seed layer hardened, the well plate was removed and forming agar wells (Dirgantara et al., 2021).

The effectiveness of brotowali (*T. crispera*) stem extract against *C. albicans* and *M. furfur* was evaluated using the agar well diffusion method. The agar wells formed by the well plate mold on Sabouraud Dextrose Agar (SDA) were filled with brotowali stem extract at concentrations of 45%, 60%, 75%, and 90%, as well as with the positive control

solution and the negative control solution. Incubation was carried out in an incubator for 2×24 hours at 30°C . Each treatment group was tested with a minimum of four replicates. Measurement of the inhibition zones was carried out using a digital caliper. Clear inhibition zones of varying diameters were observed in *C. albicans* and *M. furfur* cultures treated with the brotowali stem extract.

Data Analysis

This study used the nonparametric Kruskal–Wallis test as an alternative statistical method, followed by the Mann–Whitney post hoc test, as the data were not normally distributed and were not homogeneous. A comparison test was also carried out between the same concentrations against both fungal species. The Mann–Whitney post hoc test was used to determine differences in the antifungal effectiveness of brotowali stem extract at the same concentrations against *C. albicans* and *M. furfur*.

Result

The inhibition zones formed around the agar wells were measured and classified according to antifungal activity as weak, moderate, strong, and very strong (Davis & Stout, 2009), as presented in Table 2.

Table 2. Antifungal Activity of Brotowali Stem Extract against *C. albicans* and *M. furfur* (2×24 hours)

Concentration	<i>C. albicans</i> (mm)	Category	<i>M. furfur</i> (mm)	Category
45%	$3,63 \pm 0,05$	Weak	$2,38 \pm 0,34$	Weak
60%	$4,00 \pm 0,45$	Weak	$3,00 \pm 0,27$	Weak
75%	$4,32 \pm 0,26$	Weak	$3,28 \pm 0,24$	Weak
90%	$5,93 \pm 1,08$	Moderate	$3,97 \pm 0,22$	Weak
Positive Control	$27,35 \pm 3,04$	Very Strong	$22,70 \pm 2,41$	Very Strong
Negative Control	$0,00 \pm 0,00$	-	$0,00 \pm 0,00$	-

The Kruskal–Wallis test showed significant differences in inhibition zone diameters among extract concentrations for *C. albicans* ($p = 0.002$) and *M. furfur* ($p = 0.001$). Post hoc analysis using the Mann–Whitney test demonstrated that increasing extract concentrations generally resulted in significant differences compared with lower concentrations and the negative control ($p < 0.05$).

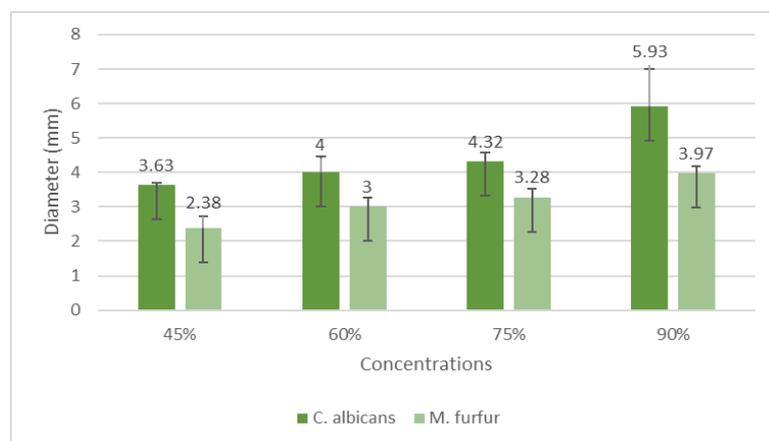


Figure 1. Comparison of the Mean Inhibition Zone Diameters of Brotowali Stem Extract against *C. albicans* and *M. furfur*.

Figure 1 illustrates a trend of increasing inhibition zone diameters with rising concentrations of brotowali stem extract against *C. albicans* and *M. furfur*. However, the observed inhibition zones were relatively small (3–6 mm), indicating weak to moderate

antifungal activity. The antifungal response against *C. albicans* appeared to be greater than that against *M. furfur* at equivalent extract concentrations.

Discussion

The results of this study showed that brotowali stem extract produced inhibition zones at all tested concentrations against both *C. albicans* and *M. furfur*. However, the diameters of the inhibition zones were relatively small, indicating that the resulting antifungal activity ranged from weak to moderate. The inhibition zones appeared as clear areas surrounding the agar wells where the brotowali stem extract was applied. The formation of these inhibition zones is caused by the presence of chemical compounds or secondary metabolites in the brotowali stem extract that are capable of inhibiting fungal growth.

Based on the phytochemical screening, the ethanol extract of brotowali (*T. crispa*) stems showed positive results for the presence of several secondary metabolites, including alkaloids, flavonoids, phenols, terpenoids (steroids), tannins, and saponins. These findings are consistent with previous studies using brotowali stem extract with 96% ethanol as the solvent, which reported the presence of flavonoids, phenols, triterpenoids, and alkaloids (Erza et al., 2020; Hutomo et al., 2022). These secondary metabolites have been widely reported to have antifungal activity and are thought to contribute to the formation of inhibition zones against the growth of *C. albicans* and *M. furfur* observed in this study.

The predominant secondary metabolites detected in the ethanol extract of brotowali (*T. crispa*) stems were alkaloids, flavonoids, and tannins. Previous studies have reported that alkaloids can disrupt fungal cell membranes through interactions with ergosterol, potentially leading to increased membrane permeability (Maisarah et al., 2023). Other studies have also shown that alkaloids may play a role in disrupting fungal cell metabolism, including the process of DNA and RNA replication (Fatma et al., 2021). At pH levels above 7, alkaloids can create an unfavorable environment for fungal growth, as fungi usually grow at a pH range of approximately 4.5–6.5, thereby inhibiting fungal proliferation (Widawati et al., 2022). In addition to alkaloids, flavonoids have been reported to exhibit antifungal activity through mechanisms that may involve the induction of mitochondrial dysfunction, disruption of plasma membrane integrity, and inhibition of replication, RNA and protein synthesis, as well as cell wall formation. Flavonoids contain hydroxyl groups that interact with fungal cell membrane phospholipids and are known to inhibit efflux pumps in fungal cells, leading to the accumulation of toxic substances (Aboody & Mickymaray, 2020; Agustina et al., 2021). Tannins are also recognized for their antifungal potential by inhibiting ergosterol biosynthesis, a key component of fungal cell membranes. Furthermore, tannins can inhibit chitin synthesis during cell wall formation, disrupt protein and nucleic acid synthesis, and suppress hyphal tip formation by inhibiting enzymes essential for fungal growth (Hersila et al., 2023). However, this study antifungal activity solely based on inhibition zone formation. Therefore, the specific mechanism of action of the active compounds in the brotowali stem extract could not be directly determined.

Administration of brotowali (*T. crispa*) stem extract against *C. albicans* after 2 × 24 hours resulted in mean inhibition zone diameters of 3.63 mm, 4.00 mm, 4.32 mm, and 5.93 mm at concentrations of 45%, 60%, 75%, and 90%, respectively. The smallest inhibition zone was observed at the lowest concentration (45%), while the highest inhibitory effect was observed at the highest concentration (90%). The concentration of a substance directly influences its antifungal effectiveness, indicating that higher concentrations of brotowali stem extract produce stronger inhibitory effects against the tested fungus (Brooks et al., 2005).

The results of this study differ from previous study that used 96% ethanol extract from brotowali stems using the reflux method, which reported no antifungal effectiveness

against *C. albicans* (Limyati et al., 1998). This can be caused by differences in extraction methods used. Reflux extraction is a heat-based method involving heating at the boiling point, which may lead to the degradation of certain heat-sensitive antifungal compounds (Azhari et al., 2020; Suprasetya, 2023). Meanwhile, in the cold method maceration tends to retain secondary antifungal metabolites that are sensitive to high temperatures.

The mean inhibition zone diameters observed after 2 × 24 hours of exposure to brotowali (*T. crispa*) stem ethanol extract against *M. furfur* at concentrations of 45%, 60%, 75%, and 90% were 2.38 mm, 3.00 mm, 3.28 mm, and 3.97 mm, respectively. The inhibition zones increased progressively from the lowest concentration (45%), with a mean diameter of 2.38 mm, to the highest concentration (90%), with a mean diameter of 3.97 mm. This pattern is consistent with previous studies evaluating the antifungal activity of brotowali stem ethanol extract against *Pityrosporum ovale* and *Trichophyton mentagrophytes*, which reported a direct association between increasing extract concentrations and enhanced fungal growth inhibition (Nuryanti et al., 2015).

Based on the antifungal classification by Davis and Stout, inhibitory activity is categorized into four levels, weak (≤ 5 mm), moderate (5–10 mm), strong (10–20 mm), and very strong (≥ 20 mm). The inhibitory activity of brotowali (*T. crispa*) stem extract against *C. albicans* was classified as weak, except at the highest concentration (90%), which demonstrated moderate antifungal activity. In contrast, the inhibitory effects of all tested concentrations of brotowali stem ethanol extract (45%, 60%, 75%, and 90%) against *M. furfur* were consistently categorized as weak.

Statistical analysis using the Kruskal–Wallis test showed significance values of 0.002 and 0.001 for *C. albicans* and *M. furfur*, respectively, indicating the presence of at least one statistically significant difference among the treatment groups for both fungal species. Post hoc analysis using the Mann–Whitney test showed that, for *C. albicans*, all treatment groups exhibited significant differences in antifungal activity ($p < 0.05$), except for comparisons between the 45% and 60% concentrations and between the 60% and 75% concentrations. Similarly, for *M. furfur*, the Mann–Whitney post hoc test revealed significant differences in antifungal activity among treatment groups ($p < 0.05$), except for the comparison between the 60% and 75% concentrations. In the context of drug formulation, the absence of significant differences between certain concentration pairs suggests that increasing the extract concentration from 45% to 60% and from 60% to 75% against *C. albicans*, as well as from 60% to 75% against *M. furfur*, does not consistently result in a meaningful enhancement of antifungal activity. Therefore, the use of extract concentrations above 45%–60% and 60%–75% for *C. albicans*, and above 60%–75% for *M. furfur*, may not provide substantial additional benefits, indicating that higher concentrations may not be necessary. On the other hand, the 90% brotowali stem extract concentration produced the largest inhibition zone diameters against both *C. albicans* and *M. furfur* in this study.

Furthermore, the Post Hoc Mann–Whitney test was conducted to determine the difference in the antifungal effectiveness of brotowali (*T. crispa*) stem extract at identical concentrations against *C. albicans* and *M. furfur*. At extract concentrations of 45%, 60%, 75%, and 90%, the corresponding p-values were 0.017, 0.020, 0.020, and 0.021, respectively. These results indicate that all identical extract concentrations exhibited statistically significant differences in antifungal activity between *C. albicans* and *M. furfur*. The presence of statistically significant differences suggests that each concentration of brotowali stem extract (45%, 60%, 75%, and 90%) produced distinct inhibitory effects on the growth of *C. albicans* and *M. furfur*. These differences did not occur by chance but represent genuine variations in antifungal activity. In other words, each extract concentration demonstrated a different level of effectiveness in inhibiting the growth of the two fungal species, indicating that extract concentration significantly influences antifungal activity, although the inhibitory strength remained relatively weak overall.

The inhibition zones formed by the ethanol extract of brotowali (*T. crispa*) stems against *C. albicans* were larger than those observed in *M. furfur* cultures, as illustrated in

Figure 1. This difference was confirmed to be statistically significant based on the results of the Mann–Whitney post hoc test. The smaller inhibition zones observed in *M. furfur* may be attributed to its thicker cell wall, covering about 26–37% of the total cell volume (Billamboz & Jawhara, 2023). A thicker cell wall may act as a barrier to the antifungal mechanisms of action of the active compounds present in brotowali stem extract. This structural characteristic may reduce the ability of active compounds such as alkaloids, flavonoids, and tannins to penetrate and disrupt the fungal cell membrane.

Conclusions

The ethanol extract of brotowali (*T. crispera*) stems has statistically significant antifungal activity against *C. albicans* and *M. furfur*. However, the relatively small inhibition zone diameters indicate that the antifungal activity remained limited under the in vitro testing conditions performed. The largest mean inhibition zone observed was 5.93 mm for *C. albicans* and 3.97 mm for *M. furfur*. Extract concentrations of 45%, 60%, and 75% exhibited weak antifungal activity against *C. albicans*, while moderate antifungal activity was observed at a concentration of 90%. Meanwhile, the entire concentration of the extract against *M. furfur* showed weak antifungal activity. Comparison of inhibition zones at the same extract concentrations between *C. albicans* and *M. furfur* showed a significant difference, with greater antifungal effect against *C. albicans*.

Declaration statement

The author reports that there is no potential conflict of interest.

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