

In vitro Antifungal Susceptibility Profile of Clinical *Cladophialophora boppii* in Malaysia

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Purpose: This study aimed to determine the antifungal susceptibility pattern of clinical *Cladophialophora boppii* isolates in Malaysia.

Patients and Methods: Eight clinical strains of the *C. boppii* were received from various Malaysian hospitals from the year 2020 until 2024. The isolates were obtained from patients with clinical presentations suggestive of cutaneous fungal infection. Their identities were determined using microscopic, macroscopic and molecular methods, specifically internal transcribed spacer (ITS) sequencing. Next, the antifungal susceptibility of amphotericin B, itraconazole, fluconazole, voriconazole, ravuconazole, posaconazole, ketoconazole, isavuconazole, flucytosine and terbinafine against the *C. boppii* were determined using broth microdilution method as outlined in the Clinical and Laboratory Standards Institute (CLSI) M38 guideline. The geometric means (GM) of minimum inhibitory concentration (MIC), MIC₅₀, and MIC₉₀ were determined for each antifungal. Subsequently, the Kruskal–Wallis test was performed to determine the significant difference observed in the median MIC values between the different antifungal groups (azole, polyene, pyrimidine and allylamine) against the isolate. The significance value was set at $p < 0.05$.

Results: The GM MIC, MIC₅₀ and MIC₉₀ of all tested antifungals except amphotericin B and fluconazole against the *C. boppii* were ≤ 0.25 $\mu\text{g/mL}$. In contrast, amphotericin B and fluconazole exhibited higher MICs ranging from 2 to 16 $\mu\text{g/mL}$. Furthermore, the Kruskal–Wallis test revealed a significant difference in the median MIC values across all antifungals, with a p -value of 4.94×10^{-5} .

Conclusion: In conclusion, all the *C. boppii* isolates in this study were susceptible to pyrimidine, allylamine, and azoles, while showing intermediate susceptibility to fluconazole and notable resistance to amphotericin B. Additionally, itraconazole and terbinafine could be recommended as the first-line therapy option, which was supported by their demonstrated efficacy (MIC ≤ 0.03 $\mu\text{g/mL}$) and clinical improvement observed in this study.

Keywords: *Cladophialophora boppii*, fluconazole, itraconazole, terbinafine, amphotericin B, Malaysia

Introduction

Cladophialophora boppii is a dematiaceous fungus mainly associated with chronic cutaneous and subcutaneous infections such as chromoblastomycosis.^{1–3} Epidemiological data specifically for the *C. boppii* and other melanized fungi are limited in Malaysia. However, among the 7740 worldwide cases of chromoblastomycosis documented over a decade, the contribution of *Cladophialophora* spp. was estimated to be 14.5%.⁴ The disease primarily affects the skin, presenting a diverse clinical spectrum of chronic, inflammatory lesions that can progress to tissue fibrosis, lymphatic obstruction, and even malignant transformation and are often accompanied by itching, pain and high recurrent tendency.^{4,5} The disease has also been reported in tropical and temperate regions including Malaysia.^{6–8} Additionally, it is often associated with occupational hazards, as these fungi can be introduced into the body through minor injuries sustained by farmers or gardeners engaged in agricultural activities.⁹

The majority of *Cladophialophora* infections were caused by *C. carrionii* and *C. bantiana*, which are known to cause chromoblastomycosis and cerebral phaeohyphomycosis respectively.^{6,10} However, the *C. boppii* is a less-studied fungal species and is often under-identified due to its rarity and the overlapping clinical manifestations with

other black fungi.¹ Although specific data on *C. boppii* are limited, infections caused by this species have been increasingly recognized.^{1–3,11} This rise in recognition can be attributed to advances in molecular diagnostics, which help differentiate it from other closely related fungi.^{2,12}

Managing chromoblastomycosis is challenging due to the variable treatment response and high recurrence rates.¹³ The treatment mainly relies on limited open trials or expert opinions which often involve itraconazole or physical methods.¹⁴ With a lack of comprehensive guidelines for treating infections caused by this fungus, antifungal susceptibility testing is crucial to guide appropriate treatment and monitor the development of drug resistance.¹⁵ Nevertheless, data on the antifungal susceptibility profile of the clinical *C. boppii* isolates in Malaysia remains scarce. Therefore, this study aims to assess the antifungal susceptibility profile of the clinical *C. boppii* isolates with cutaneous fungal infection in Malaysia, contributing valuable insights to inform treatment strategies and improve patient outcomes.

Materials and Methods

Ethics

Ethical review was conducted and approved by the Medical Research and Ethics Committee, Ministry of Health of Malaysia, Malaysia (NMRR-20-207-53067). This study also complies with the Declaration of Helsinki. The informed consent had been obtained from the study participants before the study commencement.

Isolate

From 2020 to 2024, eight clinical isolates of the *C. boppii* were received from various public hospitals in Malaysia and cultured on Sabouraud's dextrose agar (SDA). Specifically, one isolate was collected from the hospital in the northern region, four isolates each were collected from hospitals located in the central region and three isolates were collected from hospitals in the southern region. The isolates were obtained from patients with clinical presentations suggestive of cutaneous fungal infection and their identities were confirmed by macroscopic, microscopic and molecular methods.

The molecular identification was conducted using internal transcribed spacer (ITS) sequencing. The DNA of each isolate was extracted according to the instructions of the Zymoresearch Quick-DNATM Fungal/Bacterial Miniprep kit (Murphy Ave, Irvine, United States). Next, the amplification of the ITS was performed using universal primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3').^{3,16} PCR reactions were performed in a 50 µL mixture containing 25 µL of 2X MyTaq HS Master Mix (Bioline, Meridian Bioscience, Cincinnati, OH, USA), 1 µL of each primer (20 µM), 2 µL of DNA template and 21 µL of nuclease-free water. Following that, PCR was conducted with the cycling conditions of an initial denaturation at 95°C for 1 min, followed by 35 cycles of denaturation at 95°C for 15 sec, annealing at 56°C for 15 sec, and extension at 72°C for 10 sec.

Amplicons were purified using the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) and sequenced using the Sanger sequencing method. The obtained sequences were analyzed using the Basic Local Alignment Search Tool (BLAST) against the NCBI GenBank database to determine the species identity. A threshold of ≥98% identity,^{17,18} ≥99% query coverage¹⁷ and an E-value of ≤1e⁻⁵ was used for the confirmation.¹⁹

Susceptibility Testing

Due to the lack of established guidelines for susceptibility testing of *C. boppii*, the MIC was determined using the broth microdilution method as outlined in CLSI M38.²⁰ Briefly, 10–14 days-old cultures of the *C. boppii* were suspended in 0.85% saline. Next, the suspension was allowed to settle for 5 to 10 min, and a final working conidial suspension of 1×10³ to 3×10³ colony-forming unit (CFU)/mL was prepared. Subsequently, 100 µL of conidial suspension was introduced into each microdilution well.

The antifungals selected for testing were amphotericin B (0.004–8 µg/mL), itraconazole (0.001–0.5 µg/mL), fluconazole (0.125–64 µg/mL), voriconazole (0.001–0.5 µg/mL), ravuconazole (0.004–8 µg/mL), posaconazole

(0.004–8 µg/mL), ketoconazole (0.004–8 µg/mL), isavuconazole (0.004–8 µg/mL), flucytosine (0.004–8 µg/mL) and terbinafine (0.001–0.5 µg/mL). After preparing the conidial suspension, an additional 100 µL of each antifungal solution was added to the corresponding microdilution well, and the mixture was incubated at 28°C for 96 h.²¹ Two reference strains, namely *Candida parapsilosis* (ATCC 22019) and *Candida krusei* (ATCC 6258) were included as quality controls to ensure the accuracy and reliability of the results. Additionally, *Trichophyton rubrum* (ATCC MYA-4438) was used as quality control for terbinafine susceptibility testing.

Finally, the MIC was read as the lowest concentration of antifungals preventing 100% of growth (amphotericin B), or 80% of growth (all antifungals except amphotericin B) compared to the growth control.²⁰ As no breakpoint is currently established for *C. boppii*, its susceptibility can be categorized based on previous studies.²² The samples were then categorized as susceptible to isavuconazole at MIC ≤1 µg/mL and resistant at MIC ≥4 µg/mL; and susceptible to fluconazole at MIC ≤ 8 µg/mL, intermediate 8 µg/mL < MIC < 64 µg/mL, and resistant at MIC ≥64 µg/mL.^{22,23} Furthermore, the sample was classified as susceptible to other tested antifungals at MIC <1 µg/mL, intermediate at 2 µg/mL and resistant at ≥4 µg/mL.^{22,24,25}

Data Analysis

For each antifungal test, the GM MIC, MIC₅₀ and MIC₉₀ were calculated. MIC₅₀ was defined as the MIC at which 50% of the isolates were inhibited; whereas MIC₉₀ is the MIC at which 90% of the isolates were inhibited. Next, the Kruskal–Wallis test was used to determine the significant difference observed in the median MIC values between the different antifungal groups (azole, polyene, pyrimidine and allylamine) against the isolate using SPSS 20.0 (IBM®, Armonk, New York). *P*-values less than 0.05 were considered statistically significant.

Results

Patients

All patients were managed at dermatology clinics of five public hospitals. The age of patients ranged from 25 to 69 years old, with females comprising 63% (n=5) of the cases. They had various underlying medical conditions which included eczema (n=4), hypertension (n=4), diabetes mellitus (n=3), dyslipidaemia (n=3), ischaemic heart disease (n=2), cerebrovascular accident (n=1), psoriatic arthropathy (n=1) and cellulitis of the legs (n=1). The anatomical sites of involvement include the face and neck region, upper and lower limbs, trunk, feet, scalp and intertriginous area. The duration of lesions ranged between three months and eight years. The skin lesions were pruritic. The clinical diagnosis prior to laboratory confirmation included superficial cutaneous fungal infection (n=5) and subcutaneous fungal infection (n=3). The most common relevant cutaneous presentations were annular scaly plaques with center clearing (n=5), thick scaly plaques (n=4), multiple crusted plaques (n=2), and excoriated papules (n=1).

Histopathological examination was performed on three patients who consented to skin biopsy in this study. Spongiotic epidermal changes (n=2) and hyperkeratotic epidermis with papillomatosis (n=1) associated with chronic dermal inflammatory response were observed. Fungal bodies were identified at the stratum corneum using Periodic Acid-Schiff stain in two of them. The combination of clinical features, microbiological evidence, and molecular identification aligns with the diagnostic criteria for chromoblastomycosis outlined by Centers for Disease Control and Prevention and Borges et al.^{26,27}

Of the eight patients in this study, seven received either oral itraconazole, oral terbinafine, or both in combination for their treatment. The remaining one used only ketoconazole and selenium sulfide shampoo for the affected area. All experienced clinical improvement although three had recurrences.

Isolate Information

The *C. boppii* was the sole pathogen isolated from skin scrapping (n=5) and tissue biopsy (n=3). The colony is olive-grey to black while the reverse is black on SDA agar (Figure 1). The microscopic findings showed conidia were smooth-walled, subspherical and formed into a long chain acropetally from the sides of the septated hyphae.

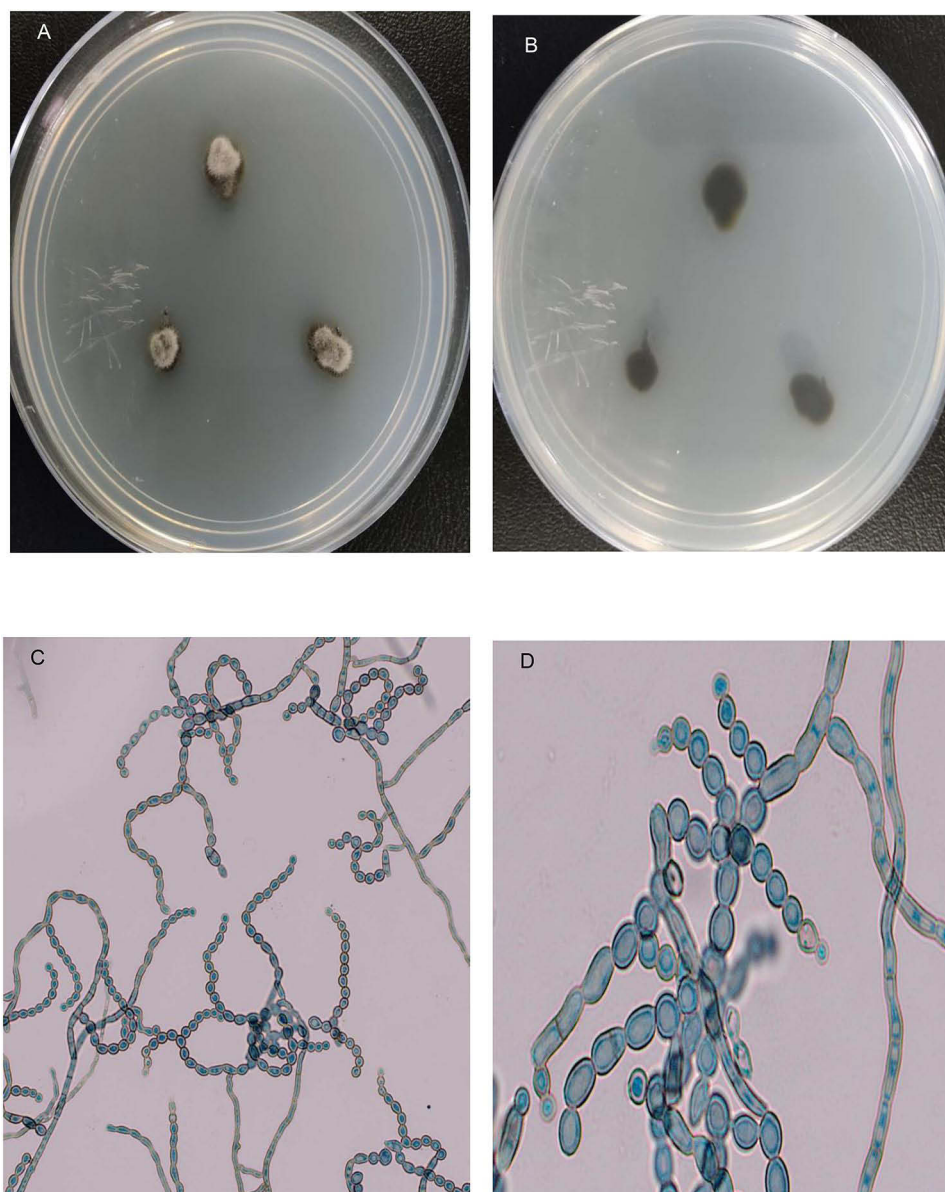


Figure 1 Macroscopic morphology of *C. boppii* on SDA (A) and reverse of SDA (B) after 14 days of incubation at 28°C. Microscopic morphology of the colony after lactophenol blue staining with magnification of 400x (C) and 1000x (D).

Molecular Identification

Molecular identification using ITS sequencing has confirmed all eight isolates as *C. boppii*. The BLAST analysis of the ITS sequences revealed a 99–100% sequence identity match to *C. boppii* sequences in the NCBI GenBank database, with E-values of 0 and query coverage ranging from 99% to 100% (Table 1).

Antifungal Susceptibility Pattern

Overall, all isolates were susceptible to pyrimidine, allylamine, and azoles except for fluconazole. While all isolates showed intermediate susceptibility to fluconazole, 87.5% exhibited resistance to amphotericin B. Fluconazole recorded the highest GM MICs at 10.08 µg/mL, followed by amphotericin B, itraconazole, voriconazole and ravuconazole, at 4.49 µg/mL, 0.06 µg/mL, 0.05 µg/mL and 0.05 µg/mL, respectively. Other antifungals, including posaconazole, ketoconazole, isavuconazole, flucytosine, and terbinafine, consistently demonstrated low MICs of 0.03 µg/mL (Table 2).

Table 1 BLAST Analysis Results for *C. boppii* Isolates

Isolate	Identity (%)	E-value	Query Coverage (%)	Reference (Accession Number)
1	100	0	99	<i>C. boppii</i> (MH062995.1)
2	100	0	100	<i>C. boppii</i> (MH062995.1)
3	99	0	100	<i>C. boppii</i> (MH876019.1)
4	100	0	99	<i>C. boppii</i> (MH062995.1)
5	100	0	100	<i>C. boppii</i> (MH876019.1)
6	100	0	100	<i>C. boppii</i> (MH729009.1)
7	100	0	100	<i>C. boppii</i> (MH725587.1)
8	100	0	100	<i>C. boppii</i> (MH062995.1)

Abbreviation: E-value, Expectation-value.

Table 2 MIC of Each Antifungal Drug Against *C. boppii* in This Study

Antifungal Group	Antifungals	MIC (µg/mL)			
		Range	GM MIC	MIC ₅₀	MIC ₉₀
Polyene	Amphotericin B	2–8	4.49	4	8
Azoles	Fluconazole	8–16	10.08	8	16
	Itraconazole	0.03–0.06	0.06	0.06	0.06
	Voriconazole	0.03–0.25	0.05	0.03	0.13
	Ravuconazole	0.03–0.13	0.05	0.03	0.13
	Posaconazole	0.03	0.03	0.03	0.03
	Ketoconazole	0.03	0.03	0.03	0.03
	Isavuconazole	0.03	0.03	0.03	0.03
	All azoles	0.03–16	0.08	0.03	8
Pyrimidine	Flucytosine	0.03	0.03	0.03	0.03
Allylamine	Terbinafine	0.03	0.03	0.03	0.03

Abbreviations: GM, geometric mean; MIC, minimum inhibitory concentration; µg/mL, micrograms per millilitre; MIC₅₀, the lowest concentration of the tested antifungal at which 50% of the isolates were inhibited; MIC₉₀, the lowest concentration of the antifungal at which 90% of the isolates were inhibited.

The MIC₅₀ reflected a similar pattern, with fluconazole showing the highest value at 8 µg/mL, followed by amphotericin B at 4 µg/mL and itraconazole at 0.06 µg/mL; the remaining antifungals maintained low MICs at 0.03 µg/mL. For MIC₉₀, fluconazole also had the highest value at 16 µg/mL, followed by amphotericin B at 8 µg/mL, voriconazole and ravuconazole at 0.13 µg/mL, itraconazole at 0.06 µg/mL and other tested antifungals at 0.03 µg/mL. The Kruskal–Wallis test revealed significant differences ($p < 0.05$) in the median MIC values among all antifungal groups.

Discussion

Cladophialophora boppii is a dematiaceous fungus with increasing clinical importance and often associated with recalcitrant infection.^{3,28} The finding of *C. boppii* as the etiologic agent of chronic cutaneous fungal infections, more specifically chromoblastomycosis, was relied on the clinical presentations, microscopic and molecular data. While the

detection of sclerotic bodies is diagnostic, it was not commonly observed in skin biopsy.^{29,30} This can also be seen in this study, where only two out of three histopathological examinations of the skin detected fungal elements, whereas both culture and molecular methods consistently supported the clinical diagnosis of all cases. These two methods are helpful in confirming the diagnosis and identifying the causative agent of chromoblastomycosis as recommended by the World Health Organization.³¹ The same approach has also been applied in previous studies.^{32–35}

The antifungal susceptibility testing revealed that all isolates were susceptible to itraconazole and terbinafine. This result may support their utility as the feasible treatment options for chromoblastomycosis. Itraconazole is fungistatic and commonly used as the first-line antifungal therapy to treat dematiaceous dermatophytes.^{36–39} Additionally, terbinafine is fungicidal and effective in treating dermatophytes involving the skin and nails.^{36,38,39} These drugs can be administered alone or in combination for more severe and recalcitrant cases which were caused by several other dematiaceous fungi.^{6,38,39} However, taking into consideration that all the isolates of *C. boppii* were susceptible to both antifungals in this study, combination therapy may not be required. This is further supported by the clinical improvement seen in patients with the use of either itraconazole or terbinafine in this study.

Posaconazole, ravuconazole and isavuconazole have emerged as a promising alternative, exhibiting low MIC₉₀ (0.03 µg/mL) and consistently highest activity against *C. boppii* isolates. This finding aligns with a systematic review which reported none of the included chromoblastomycosis etiologic agents showed resistance to these three agents.²² Moreover, voriconazole similarly demonstrated a low MIC₉₀ value, 0.13 µg/mL. This finding was lower than that of other dematiaceous fungi such as *C. carrionii* at 0.25 µg/mL,⁴⁰ *Phialophora verrucosa* at 1 µg/mL,⁴¹ and *Cladosporium* spp. at 4 µg/mL.⁴² These newer azoles may serve as effective alternatives for cases that do not respond to traditional antifungal therapies.

Likewise, the MIC of ketoconazole against the *C. boppii* was low MIC₉₀ (0.03 µg/mL). A previous study reported its combination with flucytosine had successfully treated chromoblastomycosis by the *F. pedrosoi*.⁴³ However, it presents challenges for prolonged treatment due to potential toxicity at higher doses, including hepatotoxicity.⁵

The present study also showed a low MIC of flucytosine against *C. boppii*. This result was consistent with the findings of Vermes et al⁴⁴ who suggested flucytosine was useful in treating chromoblastomycosis. Furthermore, the combination of this drug with itraconazole successfully led to the clinical and mycological remission of chromoblastomycosis by *F. pedrosoi* in three patients.⁴⁵ This combination therapy was said to reduce side effects by allowing lower doses, minimize the risk of resistance due to the shorter duration of treatment, and decrease the likelihood of relapse by enhancing the efficacy of the two drugs.⁴⁵

In contrast, the higher MIC values for amphotericin B (2–8 µg/mL) and fluconazole (8–16 µg/mL) were observed in this study. It suggested that the reduced susceptibility to these antifungal agents may limit their therapeutic utility in clinical practice. This finding was consistent with previous studies, which reported lower susceptibility to amphotericin B and fluconazole among *Cladophialophora* spp.⁴⁰ and other dematiaceous fungi such as *Rhinocladiella mackenziei* and *Fonsecaea* spp.^{40,46} In addition, the resistance pattern of amphotericin B in this study has precluded its empirical use and could be the main reason for the significant difference that existed in median MIC values across all antifungal classes. Different antifungal classes exhibited varying levels of in vitro activities against *C. boppii* as indicated by the significant difference of MIC values between the classes. This finding has extensive therapeutic implications as it suggested that certain antifungal classes such as azoles and allylamines may be more effective than others like amphotericin B in treating *C. boppii* infection.

The combination of amphotericin B therapy with terbinafine was suggested to be effective for treating chromoblastomycosis caused by other *Cladophialophora* spp. and other dematiaceous fungi such as *Fonsecaea* spp., *Exophiala* spp., *P. verrucosa* and *Veronaea botryose*.^{47,48} However, this combination may offer limited benefit for the *C. boppii* infections given the low susceptibility observed in this study. The mechanism of amphotericin B resistance in *C. boppii* is not fully understood. However, potential mechanisms described in other fungi have been reported, such as alterations in ergosterol biosynthesis pathway where mutations in genes encoding enzymes including *ERG3* (C-5 sterol desaturase) or *ERG11* (lanosterol 14 α -demethylase), could lead to reduced amphotericin B binding efficiency.^{49–51} Another possible mechanism is the upregulation of efflux pumps where amphotericin B could be actively pumped out from the fungal cell thus lowering its intracellular concentration and activity.⁵² Besides, fungal cell biofilm production may also be responsible for

amphotericin B resistance since the biofilm's extracellular matrix may prevent drug penetration and provide an environment favorable for resistance development.^{49,53,54} Further research should be conducted to elucidate the specific mechanisms of amphotericin B resistance in *C. boppii*. Identification of such mechanisms could be imperative to explain observed high MIC values and may be able to inform strategies to overcome resistance.

Surprisingly, it was observed that female patients were more affected by the *C. boppii* which contrasts with the traditionally held male predominance view.⁶ This may be influenced by various factors including increased women's involvement in agricultural or domestic work, hormonal influence on susceptibility, and access to health care. Further investigation is necessary to understand this trend. On the contrary, although itraconazole and terbinafine were the only treatments employed in the real situation, the susceptibility profile of other antifungal agents was still relevant. It provides an alternative treatment option, supports antifungal stewardship, ensures preparedness for upcoming refractory cases, and helps optimize treatment outcomes with the minimization of resistance risks.

Overall, this study had a relatively small sample size, with only eight clinical *C. boppii* isolates being analyzed. Larger multicenter studies are needed to validate the broader applicability of these findings. Additionally, complete clinical data was inaccessible as some patients were lost to follow-up after showing initial improvement. Despite these limitations, this study represents the first comprehensive analysis of the in vitro antifungal susceptibility of the *C. boppii* in Malaysia. The findings of this study expand our understanding of antifungal susceptibility patterns in *C. boppii* and showed a similar profile to those observed in other dematiaceous fungi. These similarities suggested that the treatment protocols established for other dematiaceous fungi may also be relevant to *C. boppii*.

Conclusion

In conclusion, all the *C. boppii* isolates in this study were susceptible to pyrimidine, allylamine, and azoles, while showing intermediate susceptibility to fluconazole and resistance to amphotericin B. These findings could also potentially reduce the need for extensive antifungal susceptibility testing in routine diagnostics. Furthermore, itraconazole and terbinafine could be recommended as the first-line therapy options for the management of chronic cutaneous fungal infections caused by *C. boppii*. While the diagnosis relied on clinical presentation and mycological confirmation including culture and molecular methods, further research is warranted to confirm the applicability of these results and their clinical efficacy.

Ethics Approval

An ethical review was conducted and approved by the Medical Research and Ethics Committee, Ministry of Health of Malaysia, Malaysia (NMRR-20-207-53067).

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Disclosure

The authors report no conflicts of interest in this work.

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