In vitro antifungal screening of three moss species from Southeastern Brazil against *Candida albicans*

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Abstract: Ribeiro-Silva, C.L., Almeida, C.E.A. & Maciel-Silva, A.S. (2025): In vitro antifungal screening of three moss species from Southeastern Brazil against Candida albicans Frahmia 44:1-7*.

We report the in vitro antifungal activity of *Brittonodoxa subpinnata* (≡ *Sematophyllum subpinnatum*), *Chryso-hypnum diminutivum* and *Octoblepharum albidum* against *Candida albicans*. The material was collected in a semideciduous forest fragment in Minas Gerais, Brazil. Methanolic and ethyl acetate extracts were prepared and tested using the disc diffusion assay, which produced discrete but consistent inhibition halos. Although antifungal activity has been previously documented for some moss taxa, no specific records were found for *B. subpinnata* or *C. diminutivum*, and the present data expand the information available for *O. albidum* by incorporating material from a distinct neotropical population. This contribution provides concise methodological notes and supports further chemical and microbiological investigations on neotropical mosses.

Key words: Bryophyta, Brazilian flora, antimicrobial activity, bioprospecting, agar diffusion method.

1. Introduction

Bryophytes, including liverworts, mosses and hornworts, produce a wide diversity of secondary metabolites, such as terpenoids, phenolic compounds, bibenzyls, lignans and lipophilic aromatic derivatives, many of which play ecological roles in defence, stress tolerance and signalling (Vanderpoorten & Goffinet 2009; Asakawa et al. 2013; Sabovljević et al. 2016; Ludwiczuk & Asakawa 2019; Asakawa 2025). Although liverworts are traditionally recognized for their exceptional chemical richness (Asakawa et al. 2021; Medeiros et al. 2024; Asakawa 2025), recent studies indicate that mosses, including species from the Brazilian flora, also contain bioactive compounds with potential antimicrobial, antioxidant and anti-inflammatory properties (Maciel-Silva & Lima 2019; Klegin et al. 2023 a,b; Muniz et al. 2023; Lopes & Furlan 2025).

Here, we present an in vitro antifungal evaluation of three neotropical moss species, *Brittonodoxa subpinnata* (Brid.) W.R. Buck, P.E.A.S. Câmara & Carv.-Silva (≡ *Sematophyllum*

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subpinnatum (Brid.) E. Britton), *Chryso-hypnum diminutivum* (Hampe) W.R. Buck, and *Octoblepharum albidum* Hedw., using methanolic and ethyl acetate extracts tested against *Candida albicans*, the most common human fungal pathogen and a major cause of mucocutaneous and systemic infections, particularly in immunocompromised individuals (Kim & Sudbery 2011). The rising incidence of antifungal resistance reinforces the need to identify new sources of bioactive compounds with antifungal potential. Our aim was to document the inhibition patterns and highlight methodological aspects important for future studies on tropical mosses.

2. Material and Methods

Samples (ca. 100 cm²) from corticicolous mats of *Brittonodoxa subpinnata* (≡ *Sematophyllum subpinnatum*), *Chryso-hypnum diminutivum* and *Octoblepharum albidum* were collected between 20 August and 9 September 2025 in a semideciduous forest fragment at the Pampulha campus of Universidade Federal de Minas Gerais, Belo Horizonte, Brazil (19°52'14.6"S; 43°58'49.0"W; Figure 1A). The site corresponds to secondary vegetation embedded in an urban matrix. A detailed characterization of this study site, including environmental conditions, is provided in Teixeira et al. (2025). Species identification was performed through morphological examination under a stereomicroscope and a light microscope, using Costa & Porto (2012, 2023).

For each species, air-dried gametophytes (0.217 g for *O. albidum*; 0.643 g for *B. subpinnata*; 0.752 g for *C. diminutivum*) were cleaned by ultrasonic washing in distilled water to remove particulate debris, transferred to individual glass vials, and fully submerged in solvent (methanol or ethyl acetate; approximately 150 mL, enough to cover the plant material). We initially selected a polarity gradient of solvents, methanol (polar), ethyl acetate (intermediate polarity), and n-hexane (non-polar), because bryophytes produce metabolites spanning a wide polarity range (Sarker et al. 2006). Methanol efficiently extracts medium- to high-polarity constituents such as phenolic compounds, bibenzyls, and oxygenated terpenoids, whereas ethyl acetate recovers less-polar aromatic and terpenoid metabolites. n-Hexane would predominantly extract highly lipophilic molecules such as triterpenes, sterols, unsaturated fatty acids, hopanes, and alkanones (Asakawa & Ludwiczuk 2013).

n-Hexane extracts were excluded from the analyses because the samples became contaminated during handling; however, had they been retained, inhibition halos would likely have been small or barely detectable, as the non-polar metabolites typically recovered by n-hexane show very poor diffusion in agar-based assays. Methanol and ethyl acetate remain standard solvents in bryophyte phytochemistry and together maximize the likelihood of recovering bioactive constituents (Asakawa & Ludwiczuk 2013; Ludwiczuk & Asakawa 2019).

Samples were macerated for 72 h under static conditions. These solvents are widely used in bryophyte phytochemistry due to their ability to recover medium- and low-polarity metabolites (Asakawa et al. 2013; Ludwiczuk & Asakawa 2019). Extracts were then filtered, concentrated under reduced pressure at low temperature, and stored at –20 °C until analysis (Figure 1B– G). Only extracts with adequate volume and clarity were included.

Candida albicans (ATCC 18804 strain) was maintained on Sabouraud Dextrose Agar (SDA) at 25 °C. Inocula were standardized to the 0.5 McFarland turbidity before plating. Antifungal activity was evaluated using the disc diffusion method (Veljić et al. 2008; Hudzicki 2016). The disc diffusion assay is a qualitative, non-standardized method suitable for detecting, but not quantitatively comparing, antimicrobial properties of different samples. Sterile 6 mm paper discs received 10 µL of each extract. Negative controls consisted of pure solvents, and fluconazole discs were used as positive controls (Barry & Brown 1996). Plates were incubated for 48 h at 25 °C. Inhibition halos were measured in millimeters, and all assays were performed in technical triplicate.

3. Results

All three moss species produced discrete but measurable inhibition halos against *C. albicans* (Table 1; Figure 1H–N). Methanolic extracts generally yielded slightly larger halos than ethyl acetate extracts, particularly for *Brittonodoxa subpinnata*, which showed the highest individual value (5 mm in M1). *Octoblepharum albidum* displayed highly consistent halos across replicates and solvents, whereas *Chryso-hypnum diminutivum* exhibited slightly greater variation.

Fluconazole also produced inhibition halos, although in some replicates its values were comparable to, or even lower than, those of the crude extracts, an effect that likely reflects differences in diffusion rather than true antifungal potency. Solvent controls showed no detectable activity. These patterns align with the limited diffusion typically observed for lipophilic or structurally complex bryophyte metabolites in agar-based assays.

Species / Control	M1	M2	M3	M mean ± SE	EA1	EA2	EA3	EA mean ± SE
Octoblepharum albidum (OA)	3	3	3	3 ± 0.0	2	3	3	3 ± 0.3
Brittonodoxa subpinnata (BS)	5	3	2	3 ± 0.9	3	3	2	3 ± 0.3
Chryso-hypnum diminutivum (CD)	1	2	3	2 ± 0.6	3	3	3	3 ± 0.0
Fluconazole (F)	3	2	2	2 ± 0.3	5	3	5	4 ± 0.7
Solvent control	1	1	2	1 ± 0.3	1	1	2	1 ± 0.3

Table 1. Inhibition halos (mm) produced by methanolic (M) and ethyl acetate (EA) extracts of three moss species against *Candida albicans* ATCC 18804, including fluconazole (positive control) and solvent controls. Values correspond to technical triplicates. Mean ± SE are shown for each solvent.

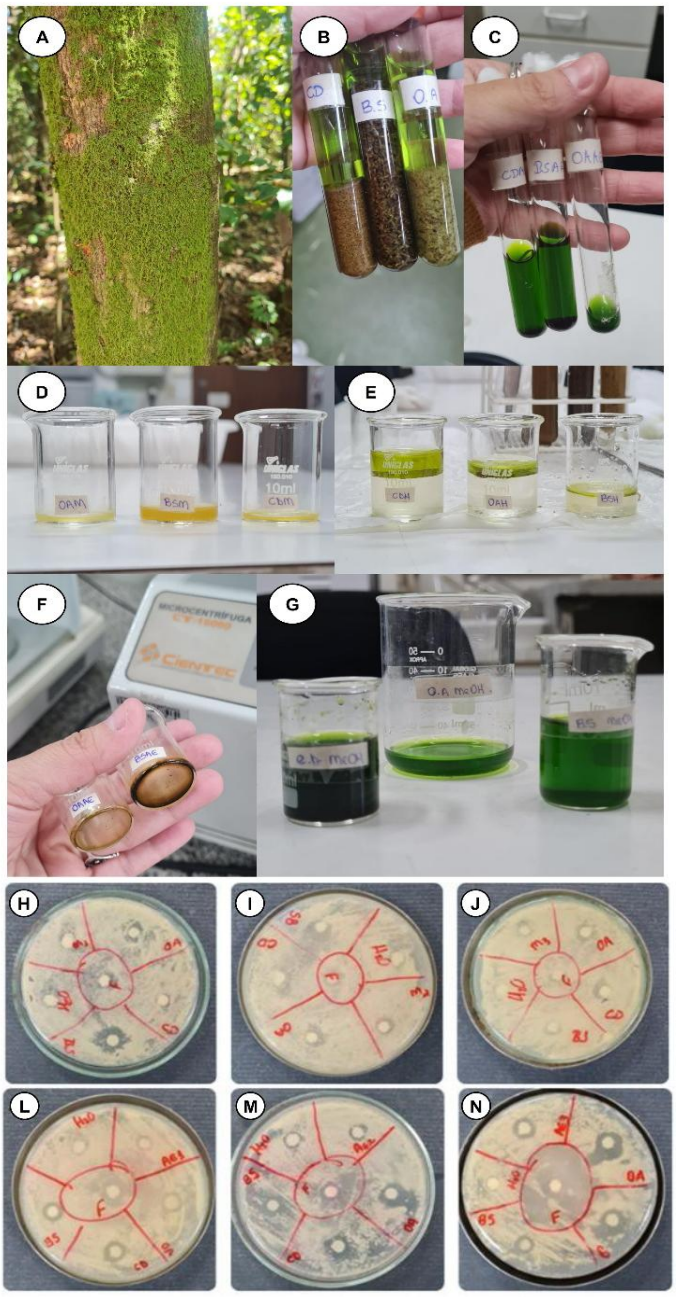

Figure 1. Workflow of collection, extraction, and antifungal assays for *Brittonodoxa subpinnata*, *Chryso-hypnum diminutivum* and *Octoblepharum albidum* against *Candida albicans*. (A) Corticicolous mat in the field. (B–C) Air-dried samples subjected to maceration in methanol and ethyl acetate. (D–G) Filtration, solvent evaporation and recovery of crude extracts. (H–N) Disc diffusion assays (48 h, 25 °C) showing inhibition halos produced by methanolic and ethyl acetate extracts of the three species, with fluconazole (F) as positive control and solvents as negative controls.

4. Discussion

The inhibition patterns observed here align with previous antimicrobial screenings of bryophytes, in which crude extracts typically produce small or barely measurable halos in agar diffusion assays (Basile et al. 1998; Bodade et al. 2008; Femi-Adepoju et al. 2013; Sabovljević et al. 2016). Such restricted halo formation is widely attributed to the poor diffusion of lipophilic or structurally complex metabolites characteristic of bryophytes, including terpenoids, phenolic acids, bibenzyls and long-chain aromatic derivatives (Asakawa et al. 2013; Manoj et al. 2016; Shete et al. 2024; Asakawa 2025) Importantly, these compounds may still display measurable antimicrobial activity even when their physical diffusion through agar is limited. Owing to the limited diffusibility of structurally complex or lipophilic natural extracts, this assay often yields only discrete inhibition halos (Bodade et al. 2008; Sabovljević et al. 2016).

The slightly greater inhibition observed in methanolic extracts is chemically consistent with established extraction procedures for bryophytes. Bryophytes produce secondary metabolites spanning a wide polarity range, and methanol is routinely used to obtain more hydrophilic and medium-polarity constituents, including phenolic compounds and simple bibenzyls, whereas nonpolar and less-polar solvents such as n-hexane, diethyl ether, methylene chloride or ethyl acetate preferentially recover lipophilic terpenoids and other hydrophobic metabolites (Asakawa & Ludwiczuk 2013). In agar-based disc diffusion assays, however, the diameter of inhibition halos depends strongly on the ability of compounds to diffuse through an aqueous medium. Extracts enriched in more hydrophilic or medium-polarity molecules tend to diffuse more efficiently and therefore form larger halos, while lipophilic constituents, although often bioactive, show limited mobility and generate very small or barely measurable zones of inhibition (Veljić et al. 2008; Basile et al. 1998; Bodade et al. 2008; Femi-Adepoju et al. 2013). Thus, the solvent-dependent differences observed here are consistent with bryophyte-specific extraction protocols and with the known limitations of agar-based diffusion assays.

Nevertheless, inhibition halo size must be interpreted with caution. A highly diffusible but weakly active extract may yield a larger halo than a potent but poorly diffusible compound (Veljić et al. 2008). This also explains why fluconazole occasionally produced halos similar to, or even smaller than, those of the crude extracts, despite its well-known efficacy against *Candida albicans* (Barry & Brown 1996). In such cases, differences in halo size reflect diffusion dynamics rather than true biological potency. Thus, similarities between fluconazole and moss extracts should not be viewed as equivalent antifungal activity, but as an artefact of how chemically complex plant metabolites spread through agar. Even so, the comparison underscores that the extracts contain constituents capable of producing measurable inhibition under diffusion-limited conditions (Basile et al. 1998; Bodade et al. 2008; Femi-Adepoju et al. 2013).

Antifungal activity has been reported for multiple moss taxa (Bodade et al. 2008; Savaroglu et al. 2011; Femi-Adepoju et al. 2013; Manoj et al. 2006), including occasional mentions of *Octoblepharum albidum* in broad-spectrum antimicrobial surveys. However, we found no previous records of antifungal assays for *Brittonodoxa subpinnata* (≡ *Sematophyllum subpinnatum*) or *Chryso-hypnum diminutivum* against *C. albicans*. Our study therefore expands the available information on neotropical bryophytes by documenting inhibition patterns for two species with no prior antifungal evaluation, while also providing data from a southeastern Brazilian population of *O. albidum*.

While disc diffusion offers a rapid first indication of antimicrobial activity, it remains a qualitative method (Barry & Brown 1996; Hudzicki 2016). Quantitative approaches, particularly broth microdilution and minimum inhibitory concentration (MIC) determination, are required to overcome diffusion limitations and accurately assess antifungal potency (Asakawa & Ludwiczuk 2013; Sabovljević et al. 2016; Kaur et al. 2023). Integrating MIC assays with metabolite fractionation and chromatographic profiling, as demonstrated in recent Brazilian bryophyte studies

(Muniz et al. 2023), will be essential for identifying active constituents and elucidating their structural and ecological roles.

In summary, although the inhibition halos observed here were small, they were consistent across species, solvents and replicates. These results underscore the potential of tropical mosses as promising, yet still largely overlooked, sources of antimicrobial metabolites. They also draw attention to key methodological aspects relevant to bryophyte bioprospecting and reinforce the need for broader phytochemical and microbiological investigations on neotropical mosses, particularly within the megadiverse Brazilian flora.

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