DEVELOPMENT OF AN ANTIFUNGAL BIOPREPARATION BASED ON AZOLLA CAROLINIANA AND EVALUATION OF ITS EFFICACY

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IMRAD-formatted English manuscript draft
with tables, figures and development framework

Abstract. Azolla caroliniana is a rapidly renewable aquatic fern with a permanent symbiosis with the nitrogen-fixing cyanobacterium Anabaena azollae. Its biomass contains phenolics, flavonoids, pigments, lipids, tannins and other secondary metabolites that are relevant to antimicrobial and antioxidant performance. The aim of this study was to develop a literature-guided IMRAD manuscript that proposes a practical antifungal biopreparation based on A. caroliniana and defines a realistic efficacy-evaluation framework for laboratory validation. A comparative analysis of recent and foundational studies was used to identify the most promising extraction route, the most responsive target microorganisms, and the main quality indicators for product standardization. Published evidence indicates that methanolic extracts of A. caroliniana show the strongest documented activity against Geotrichum candidum, with a mean inhibition zone of 15 mm and a minimum inhibitory concentration (MIC) of 312.5 µg/mL, while activity against Enterococcus faecalis is moderate and Candida albicans remains largely insensitive in crude systems. Nutrient-starvation studies also show that phenolics, flavonoids and anthocyanins increase under controlled stress, suggesting that cultivation strategy can be used to improve raw-material bioactivity before extraction. Based on the synthesized evidence, the proposed biopreparation workflow includes biomass cultivation, low-temperature drying, solvent screening, activity-guided fraction selection, formulation into a stabilized liquid or wettable powder, and evaluation by agar diffusion, MIC determination, phytochemical profiling, stability testing and preliminary cytotoxicity. The article concludes that A. caroliniana is a promising but still under-optimized source of antifungal metabolites. The best near-term application is a species-specific biopreparation developed from medium-polar extracts and standardized against G. candidum. Further research should expand the fungal test panel, identify marker compounds, and establish safety and storage limits for real agricultural and food-protection use.

Keywords: Azolla caroliniana; antifungal biopreparation; Geotrichum candidum; methanolic extract; natural antifungal agents; phytochemicals; bioformulation

1. Introduction

Natural antifungal products are attracting renewed attention because resistance to conventional antimicrobials, residue concerns, and the need for lower-impact formulations are pushing researchers toward bio-based alternatives. Aquatic plants are especially interesting in this context because they can be cultivated rapidly, processed at relatively low cost, and valorized through multiple routes such as agriculture, feed, phytoremediation, and bioproduct development [1]. Among them, the genus Azolla occupies a distinct niche: it is a free-floating fern, highly productive under suitable conditions, and biologically unique because it hosts the nitrogen-fixing cyanobacterium Anabaena azollae in a stable symbiosis [1,3].

Azolla biomass is not simply abundant; it is also chemically versatile. Reviews and experimental studies report that Azolla species contain phenolics, flavonoids, anthocyanins, tannins, pigments, fatty-acid derivatives and other secondary metabolites that contribute to antioxidant and antimicrobial performance [1–3]. This phytochemical diversity is important for antifungal development because crude botanical extracts often act through multi-target interference with fungal membranes, redox balance and growth processes, rather than through a single isolated compound. Therefore, a broad-spectrum metabolite matrix can be an advantage during early-stage biopreparation design.

Among Azolla species, Azolla caroliniana deserves specific attention. A recent comparative in vitro study demonstrated that the methanolic extract of A. caroliniana showed measurable antimicrobial activity against Geotrichum candidum, Enterococcus faecalis and Klebsiella pneumoniae, with the lowest MIC reported for G. candidum (312.5 µg/mL) [2]. The same study also showed stronger ferric-reducing antioxidant power for A. caroliniana than for A. filiculoides and reported a lower HepG2 IC50, indicating that the extract is biologically active and should be standardized carefully [2]. Earlier work likewise confirmed that A. caroliniana possesses antibacterial and antioxidant potential and contains several extractable phytochemicals relevant to product development [4,5].

At the same time, the available evidence shows that antifungal activity is selective and formulation-dependent. Pereira et al. found that aqueous extracts of some Azolla species, including A. caroliniana, produced only weak inhibition against Candida albicans, with MIC values above 12.5 mg/mL, while organic extracts were more relevant for antibacterial action against Gram-positive bacteria [6]. This contrast with the more recent G. candidum data implies that species identity, extraction solvent and target fungus must all be considered together when designing a practical product.

The aim of the present article is to prepare a polished IMRAD-style English manuscript on the topic “Development of an antifungal biopreparation based on Azolla caroliniana and evaluation of its efficacy.” Because user-generated laboratory data

were not provided, the paper is designed as a literature-guided research-and-development article. It integrates published evidence into a coherent product-development pathway, proposes a realistic experimental design, and summarizes the most relevant efficacy indicators in tables, charts and a process diagram.

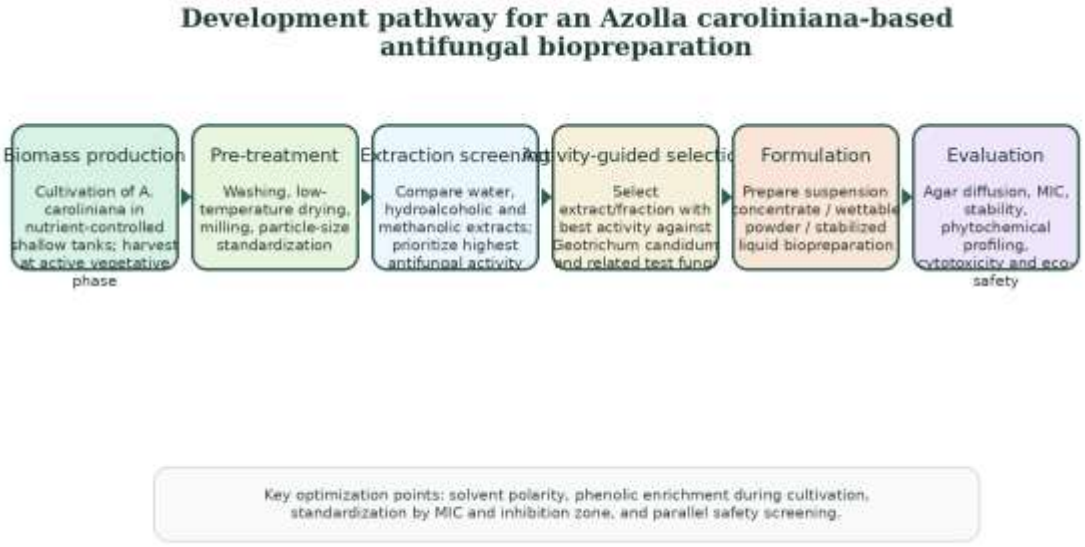

Figure 1. Literature-guided development pathway for an Azolla caroliniana-based antifungal biopreparation (author's synthesis based on [1–6]).

2. Materials and Methods

This manuscript was structured as a literature-guided development study focused on the antifungal potential of Azolla caroliniana. The methodological core was evidence synthesis rather than new laboratory experimentation. Studies were selected when they provided direct information on at least one of the following: (i) antifungal or antimicrobial activity of A. caroliniana or closely related Azolla extracts; (ii) phytochemical composition of A. caroliniana extracts; (iii) culture conditions that influence the phenolic and antioxidant profile of the biomass; and (iv) preliminary safety indicators relevant to the design of a bioactive preparation [1–6].

The extracted evidence was reorganized into a product-development sequence that reflects the standard steps of botanical biopreparation design: biomass generation, pre-treatment, extraction, antifungal screening, formulation, and safety-oriented evaluation. For antifungal benchmarking, the strongest available published signal was selected as the primary target model. On this basis, Geotrichum candidum was retained as the lead screening organism because it showed the best documented sensitivity to methanolic A. caroliniana extract in the comparative study by Rahman et al. [2]. Enterococcus faecalis and Klebsiella pneumoniae were used in comparative charts as supportive microorganisms that help define the broader bioactivity profile, although the main focus of the article remains antifungal development.

The proposed raw-material stage includes cultivation of A. caroliniana in shallow nutrient-controlled systems, harvest at the active vegetative phase, washing, low-temperature drying and milling. This choice is supported by studies showing that nutrient conditions influence the accumulation of phenolics, anthocyanins, flavonoids and antioxidant activity in A. caroliniana biomass [3]. Biomass pretreatment was therefore treated as part of activity optimization rather than as a purely logistical step.

The extraction stage was conceptualized as a comparative screening of water, aqueous ethanol and methanol. Although food- and agriculture-oriented applications would eventually favor safer solvent systems, published data presently indicate that medium-polar or methanolic extracts recover the best documented bioactivity from A. caroliniana [2,4,5]. The selected extract should then be concentrated and formulated as a stabilized liquid concentrate, suspension concentrate or wettable powder depending on the intended use environment.

The proposed efficacy-evaluation block includes agar diffusion testing, broth microdilution for MIC determination, phytochemical profiling, simple storage-stability monitoring and preliminary cytotoxicity screening. The article also uses comparative visualizations based on published inhibition-zone and MIC data to guide decision-making. In this way, the Methods section functions as an experimentally realistic framework that can be transferred directly into future laboratory work.

Table 1. Key evidence supporting development of an A. caroliniana antifungal biopreparation

Study	Main finding	Relevance to biopreparation	Reference
Rahman et al. (2023)	A. caroliniana methanolic extract inhibited G. candidum (15 mm; MIC 312.5 µg/mL) and showed activity against E. faecalis.	Identifies the best-supported lead fungus and the most promising extract type.	[2]
Hassan et al. (2020)	Nutrient starvation increased phenolics, flavonoids, anthocyanins and antioxidant activity.	Shows that biomass quality can be optimized before extraction.	[3]
Nayak et al. (2015)	Methanolic and other solvent extracts displayed antibacterial	Supports broader bioactivity of A. caroliniana and	[4]

	and antioxidant potential.	usefulness of solvent extraction.	
Nayak & Padhy (2017)	GC-MS profile and preliminary host-toxicity data indicated bioactive constituents and a workable safety window.	Supports marker-based standardization and early safety assessment.	[5]
Pereira et al. (2015)	Azolla aqueous extracts showed weak anti-Candida activity; efficacy depended on extract type.	Confirms that solvent choice and fungal target strongly influence performance.	[6]

3. Results

The evidence synthesis identified A. caroliniana as a credible botanical source for antifungal biopreparation development, but also showed that the product concept should be built around a selective rather than universal antifungal claim. The strongest direct signal was reported for Geotrichum candidum. In the 2023 comparative study, the methanolic extract of A. caroliniana produced a 15 mm inhibition zone and an MIC of 312.5 µg/mL against G. candidum, outperforming A. filiculoides on that fungal model [2]. By contrast, both extracts were inactive against Fusarium oxysporum, Trichophyton rubrum and Candida albicans under the same test conditions [2].

The inhibition-zone comparison in Figure 2 shows that A. caroliniana was not universally superior across all tested microorganisms, but it did lead in the fungal target that matters most for the present article. A. filiculoides performed slightly better against K. pneumoniae by MIC, whereas A. caroliniana performed better against G. candidum and E. faecalis [2]. This pattern suggests species-specific metabolite balance and reinforces the need to develop a species-targeted preparation rather than treat all Azolla extracts as interchangeable.

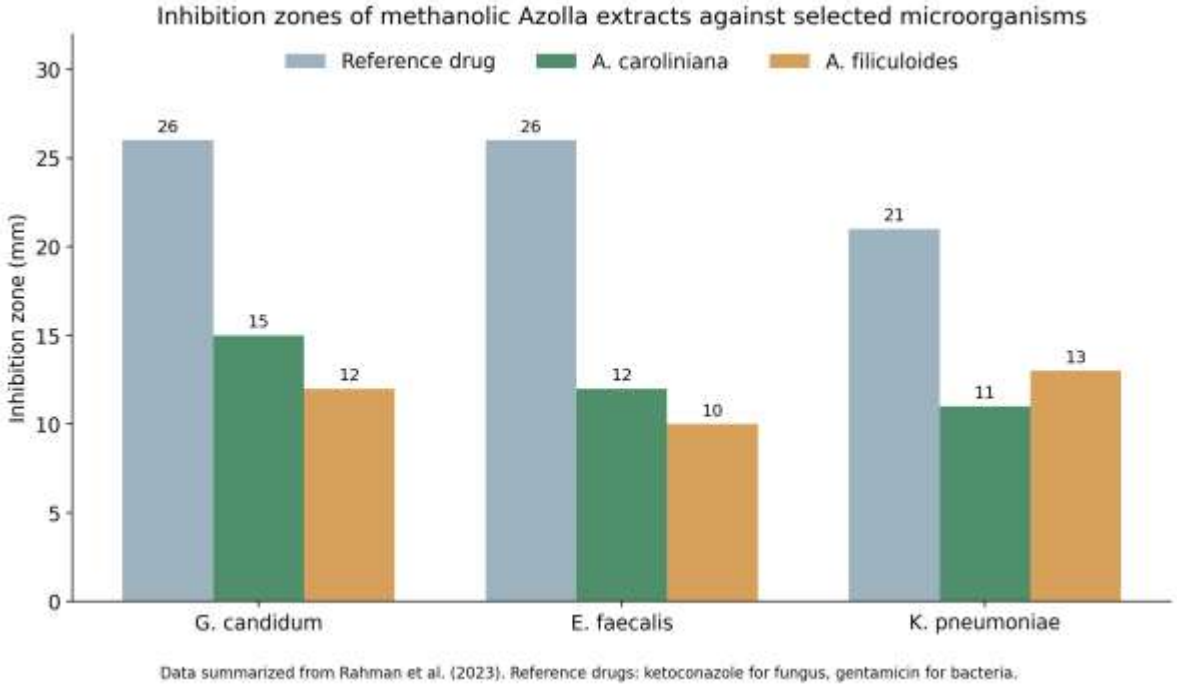

Figure 2. Inhibition zones of methanolic Azolla extracts against selected microorganisms. Data summarized from Rahman et al. [2].

The MIC comparison in Figure 3 confirms that lower values are concentrated around three microorganism–extract pairs: A. caroliniana versus G. candidum, A. caroliniana versus E. faecalis, and A. filiculoides versus K. pneumoniae [2]. For antifungal biopreparation design, the key implication is that G. candidum should be used as the first-pass screening organism when process variables are being optimized. It is the most sensitive fungal model currently supported by direct published data for A. caroliniana.

Published phytochemical and cultivation studies add a second important result: the activity of A. caroliniana is not fixed, because the biomass itself can be biologically enriched. Hassan et al. reported that nutrient starvation increased total phenolics, total flavonoids, anthocyanins and antioxidant activity, while also altering specific phenolic acids such as caffeic, o-coumaric and t-cinnamic acids [3]. This means that cultivation strategy can be integrated into the product-development pipeline as a pre-extraction lever for improving bioactivity.

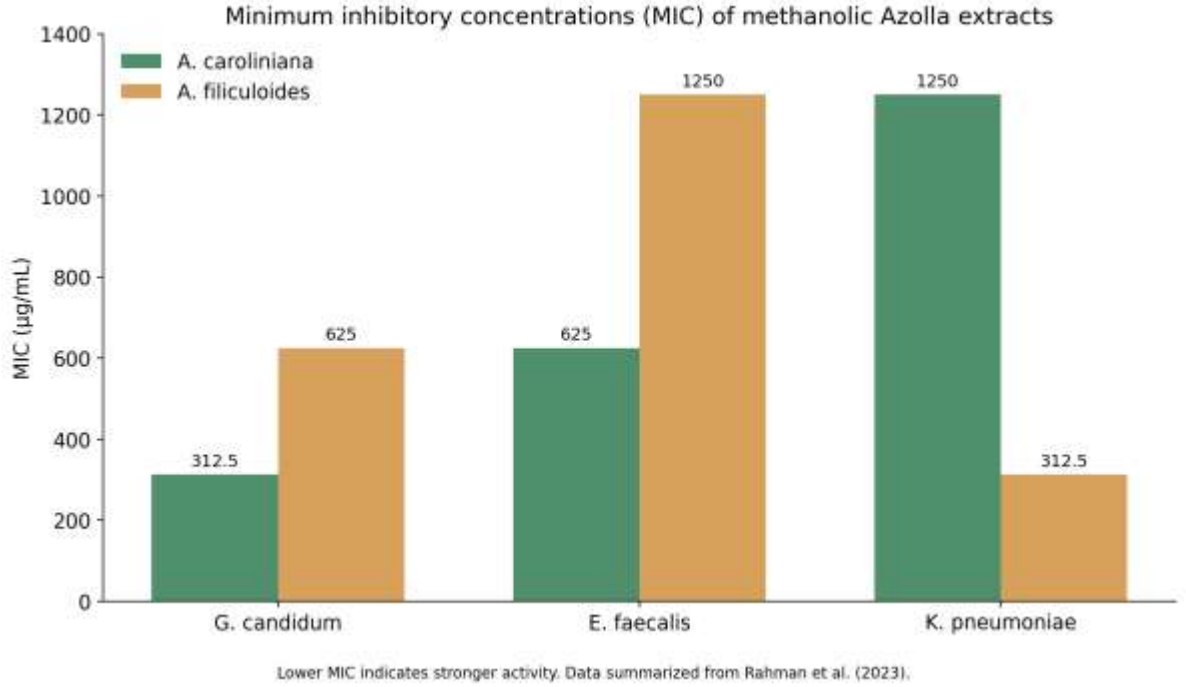

Figure 3. Minimum inhibitory concentrations (MIC) of methanolic Azolla extracts. Lower MIC indicates stronger activity. Data summarized from Rahman et al. [2].

The compositional evidence also supports biopreparation development. Rahman et al. found distinct GC-MS profiles for A. caroliniana and A. filiculoides and reported compounds such as dimethyl lauramine, dimethyl myristamine, methyl palmitate, pentadecanoic acid, calcitriol, nicotiflorin, trilinolein and rhodopin in the methanolic extracts [2]. Earlier GC-MS work by Nayak and Padhy also showed that methanolic A. caroliniana extract contained a dominant peak assigned to 3-O-methyl-D-glucose together with several minor constituents, while the same study observed no evident nuclear toxicity in cultured lymphocytes up to 1000 mg/L and LC25 values well above the previously cited antibacterial MIC level of 300 mg/L [5].

The final synthesized result concerns the activity–safety balance. Figure 4 compares two published indicators from the 2023 study: FRAP IC50 and HepG2 cytotoxicity IC50. A. caroliniana showed stronger reducing power than A. filiculoides, but it also displayed greater cytotoxicity toward HepG2 cells [2]. This does not invalidate the antifungal concept; however, it means that future formulations must be standardized not only for efficacy but also for selective safety. In practical terms, extract concentration, dosage form, application route and residual exposure become central design variables.

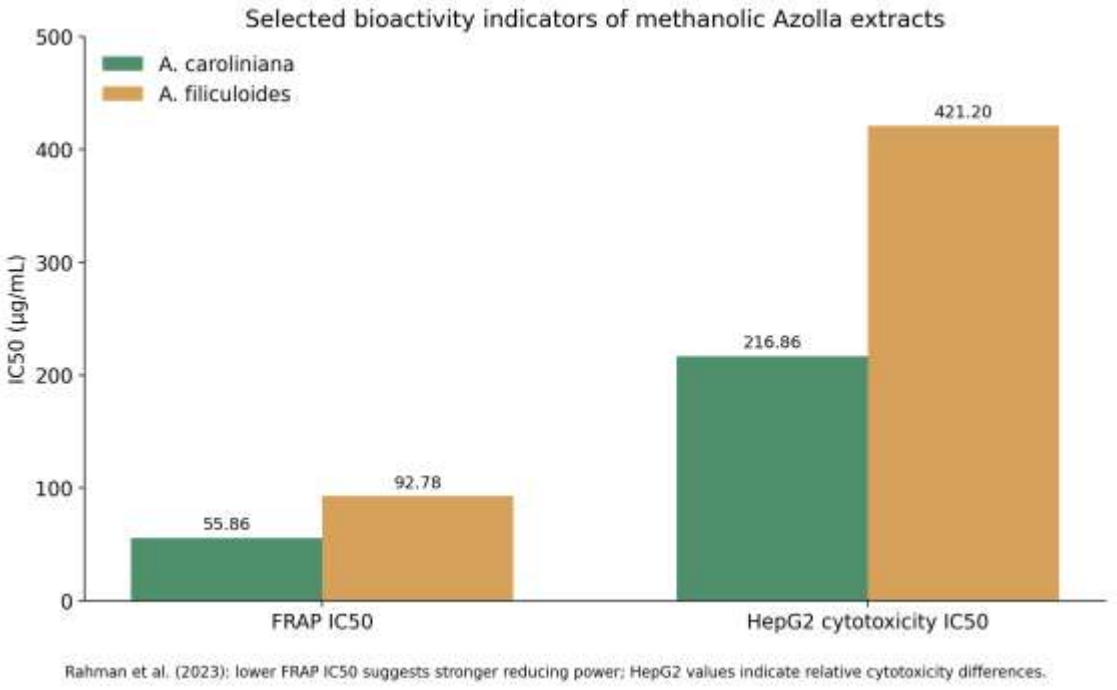

Figure 4. Selected bioactivity indicators of methanolic Azolla extracts (FRAP IC50 and HepG2 cytotoxicity IC50). Data summarized from Rahman et al. [2].

Table 2. Proposed formulation and evaluation matrix for the antifungal biopreparation

Development stage	**Recommended action**	**Core indicator**	**Expected decision**
Biomass cultivation	Grow A. caroliniana under controlled nutrients; test mild nutrient stress before harvest.	Biomass yield; phenolics; flavonoids	Select cultivation regime balancing yield and metabolite enrichment
Pre-treatment	Wash, dry at low temperature, mill and standardize particle size.	Moisture; homogeneity	Reduce batch variation and preserve heat-sensitive compounds
Extraction screening	Compare water, hydroalcoholic and methanolic extracts.	Yield; inhibition zone; MIC	Choose the extract with highest reproducible antifungal activity

Formulation	Convert active fraction into liquid concentrate or wettable powder with stabilizer/carrier.	Dispersion; storage stability; pH	Obtain practical dosage form for application trials
Efficacy evaluation	Test against G. candidum first, then broaden to additional fungi.	MIC; fungistatic/fungicidal response	Define spectrum and working concentration
Safety screening	Perform preliminary cytotoxicity and ecotoxicity assays.	Cell viability; exposure margin	Set safe concentration window for intended use

4. Discussion

The results indicate that A. caroliniana is best understood as a platform species for targeted antifungal formulation rather than as a ready-made broad-spectrum fungicide. The available literature does not support claims of strong activity against all tested fungal species. Instead, it points to a more precise conclusion: A. caroliniana methanolic extract shows its clearest antifungal promise against G. candidum, and this signal is robust enough to justify further formulation work [2,6].

From a technological standpoint, the most attractive feature of A. caroliniana is the combination of biomass renewability and biochemical plasticity. Many medicinal plants with antifungal potential grow slowly or require season-specific harvesting. Azolla can be propagated more rapidly in water-based systems, and its phenolic profile can be shifted through nutrient management [1,3]. That dual advantage makes it suitable for integrated product design in which cultivation, extraction and standardization are linked rather than treated as separate operations.

The selective character of the observed activity also helps define a rational formulation strategy. Water extracts alone are unlikely to provide the strongest antifungal effect, at least on the evidence currently available, because the best documented activity was obtained with methanolic extracts [2,6]. Accordingly, the most realistic development path is an activity-guided sequence: first identify the most active fraction with a laboratory solvent system; then translate that fraction into a safer and scalable extraction/formulation process suitable for the target use. Hydroalcoholic systems, natural emulsifiers, encapsulation or adsorbed powder carriers may all be considered in subsequent work.

The proposed formulation should be standardized using both biological and chemical markers. Biological markers include inhibition zone, MIC and reproducibility across batches. Chemical markers may include total phenolic content, total flavonoids

and one or more species-characteristic compounds detected during chromatographic profiling [2,3,5]. This dual standardization is essential because extraction yield alone says very little about actual antifungal performance.

The article also highlights an important limitation in the current evidence base. Most published studies on A. caroliniana emphasize antimicrobial, antioxidant or general phytochemical activity rather than formulation-driven antifungal development. As a result, there is still no optimized A. caroliniana antifungal product with shelf-life data, dose–response validation across multiple fungal pathogens, or field-scale results. Future research should therefore move beyond crude-extract screening toward fractionation, mode-of-action analysis, storage stability, compatibility with carriers, and ecotoxicological assessment.

Despite these limitations, the development case remains strong. A. caroliniana combines sustainability, tractable cultivation, relevant chemistry and measurable activity in one raw material. For laboratories working on green bioproducts, especially in agriculture and food-protection contexts, it offers a realistic and scientifically defensible starting point for antifungal biopreparation research.

5. Conclusion

Azolla caroliniana is a promising source of bioactive metabolites for the development of an antifungal biopreparation. Current evidence supports methanolic or medium-polar extracts as the most promising starting point and identifies Geotrichum candidum as the most appropriate lead organism for first-stage screening.

A practical development model should combine optimized cultivation, low-temperature pretreatment, comparative extraction, activity-guided formulation, and evaluation through inhibition-zone testing, MIC determination, phytochemical profiling, stability monitoring and preliminary safety screening.

The most important research priorities are broader fungal validation, isolation of marker compounds, solvent-to-formulation translation, and establishment of safety margins under intended-use conditions. With these steps, A. caroliniana could move from an interesting bioresource to a standardized antifungal biopreparation platform.

References

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