

Comparative In Vitro Antifungal Activity of *Artemisia herba-alba*, *Haloxylon articulatum*, and Their Co-Hydrodistilled Formulations

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Abstract

This study evaluated the in vitro antifungal activity of essential oils obtained from *Artemisia herba-alba* and *Haloxylon articulatum*, individually and after co-hydrodistillation at three plant ratios. The preparations were tested against *Aspergillus niger*, *Penicillium expansum*, *Fusarium oxysporum* f. sp. *albedinis*, *Rhizopus stolonifer*, and *Candida albicans* using direct-contact/radial-growth and diffusion-based assays. *A. herba-alba* produced a higher essential-oil yield than *H. articulatum* (12.38% vs 1.30%) and showed the broadest individual-preparation antifungal profile. In direct-contact assays, complete visible inhibition of *F. oxysporum*, *R. stolonifer*, and *C. albicans* was observed from 0.007 µL/mL, whereas *A. niger* remained visibly growing across the tested concentration range. The co-hydrodistilled preparations showed formulation-dependent responses: M1 inhibited *F. oxysporum* and *C. albicans* from 0.007 µL/mL, M2 generally required higher tested concentrations for complete inhibition, and M3 inhibited *C. albicans* from 0.008 µL/mL. Apparent susceptibility also varied across assay formats, particularly for *A. niger*, which showed persistent growth in some direct-contact conditions but measurable inhibition zones in diffusion assays. The findings indicate that antifungal performance depended jointly on botanical source, co-hydrodistilled formulation, fungal species, concentration, and assay format. Given the descriptive design and absence of chemical characterization, these findings should be interpreted as exploratory rather than mechanistic or synergistic evidence.

Keywords: *Artemisia herba-alba*; *Haloxylon articulatum*; essential oils; co-hydrodistillation; antifungal activity; *Candida albicans*; *Aspergillus niger*; assay dependence

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1. Introduction

Fungal contamination and fungal disease affect food quality, crop production, stored products, and human health, while the search for plant-derived antifungal agents continues to attract attention as an alternative or complementary strategy to conventional fungicides and antifungal drugs. Essential oils are of particular interest because their volatile and lipophilic constituents can inhibit fungal growth through several physicochemical and cellular processes, while biological performance varies substantially across plant species, chemotypes, fungal targets, and assay conditions (Nazzaro et al., 2017; Mutlu-Ingok et al., 2020; Rashed et al., 2021). Comparative work has also shown that essential-oil susceptibility is frequently species-specific rather than uniform (Powers et al., 2019). An oil that performs well against one fungus or in one assay therefore cannot automatically be assumed to show the same activity against another organism or under another test format.

Among aromatic plants from arid and semi-arid regions, *Artemisia herba-alba* has received considerable attention because of its traditional uses, chemical

diversity, and documented antimicrobial activity. Essential oils from this species have shown activity against yeasts, molds, dermatophytes, and phytopathogenic fungi (Mighri et al., 2010; Abu-Darwish et al., 2015; Houti et al., 2023; Lazrega et al., 2025). In Algeria, recent work has also reported antifungal activity of *A. herba-alba* essential oil against *Fusarium oxysporum* f. sp. *ciceri* (Lazrega et al., 2025), while essential oils from other Algerian aromatic plants have shown activity against *F. oxysporum* f. sp. *albedinis*, confirming the relevance of essential-oil screening against date-palm and crop pathogens in the regional context (Boukhobza et al., 2021).

At the same time, *A. herba-alba* cannot be treated as chemically uniform. Essential oils obtained from Algerian, Jordanian, Tunisian, and Moroccan material have shown substantial differences in yield and major constituents (Dob & Benabdelkader, 2006; Hudaib & Aburjai, 2006; Mighri et al., 2010; Ouguirti et al., 2021). More recent seasonal work has likewise demonstrated that harvest timing can affect essential-oil yield, composition,

and antifungal activity (Elwardani et al., 2024). Accordingly, biological activity should be interpreted in relation to the specific plant material and experimental conditions rather than extrapolated across all samples of the species.

The evidence base is less developed for *Haloxylon articulatum*, particularly for directly comparable essential-oil antifungal assays. Recent work has nevertheless demonstrated that *H. articulatum* contains biologically active metabolites and that methanolic extracts can inhibit selected phytopathogenic fungi (Lakhdari et al., 2024). Such findings indicate antifungal potential at the plant level, but they do not establish that a hydrodistilled essential oil will express the same activity, because hydrodistillation and solvent extraction recover different chemical fractions. Related literature reported under *Hammada scoparia* has also produced limited antifungal responses in some essential-oil systems (Dríoiche et al., 2020). This distinction separates the broader antifungal potential of the plant from the activity of a particular hydrodistilled preparation.

Most research on combinations of essential oils concerns post-extraction mixtures, where two independently produced oils are blended and then evaluated for additive, antagonistic, or synergistic effects. Formal synergy, however, requires an interaction design such as checkerboard testing and fractional inhibitory concentration analysis rather than simple observation of stronger inhibition in a mixture (Bassolé & Juliani, 2012). Co-hydrodistillation represents a different experimental process because the two plant materials are distilled simultaneously. Evidence from other plant systems shows that a co-distilled product can differ from a simple blend of separately extracted oils and can exhibit a distinct biological profile (Nebié et al., 2023). More recent work has likewise shown that co-distillation can produce a volatile composition distinct from the corresponding parent oils and their post-extraction mixture (Nebié et al., 2024). These findings support treating co-hydrodistillation as a distinct formulation process, although antifungal evidence for the specific *A. herba-alba*–*H. articulatum* combination remains limited.

A second methodological issue concerns how antifungal activity is measured. Essential oils are hydrophobic and volatile, and their apparent activity can change with the mode of exposure. Donaldson et al. (2005) found only weak correspondence between dilution and agar-diffusion estimates for essential oils, while Tullio et al. (2007) reported clear differences between liquid and vapor-phase antifungal activity. Cavanagh (2007) similarly emphasized the difficulty of comparing essential-oil studies when volatilization and assay conditions differ. More recent reviews have reiterated that disc diffusion, well diffusion, agar incorporation, and broth-based methods do not necessarily yield interchangeable estimates because different constituents

disperse and diffuse differently (Rashed et al., 2021). Bouddine et al. (2012) demonstrated this specifically for *Aspergillus niger*, with marked differences among broth dilution, disc diffusion, and micro-atmosphere methods. Using more than one assay can therefore reveal methodological heterogeneity that a single test format may obscure.

Against this background, the present study asks: How does the antifungal activity of essential oils obtained from *A. herba-alba* and *H. articulatum*, individually and through co-hydrodistillation at different plant ratios, vary across fungal species and assay methods? The study compares the two single-plant essential oils with three co-hydrodistilled preparations produced at different AHA:HA plant ratios. Antifungal activity was evaluated against *Aspergillus niger*, *Penicillium expansum*, *Fusarium oxysporum* f. sp. *albedinis*, *Rhizopus stolonifer*, and *Candida albicans* using direct-contact/radial-growth and diffusion-based assays.

The study makes three related contributions. First, it evaluates individual and co-hydrodistilled preparations within the same experimental framework, allowing observed response profiles across plant ratios to be compared without equating them with formal synergism. Second, it treats co-hydrodistillation as distinct from post-extraction oil blending, thereby aligning the biological interpretation with the actual preparation process. Third, it preserves assay-dependent and species-dependent heterogeneity as part of the scientific result rather than collapsing all measurements into a single potency ranking. This framing provides a more precise basis for interpreting the antifungal potential of the two plants and for designing future replicated, chemically characterized combination studies. An overview of the study design and the main comparative findings is provided in the Graphical Abstract.

2. Materials and Methods

2.1. Study design and plant material

This exploratory in vitro study evaluated the antifungal activity of essential oils obtained from *Artemisia herba-alba* and *Haloxylon articulatum*, both as single-plant preparations and as co-hydrodistilled plant combinations. The experimental work was conducted in the teaching laboratory of the Department of Biology, Faculty of Natural and Life Sciences, Tahri Mohammed University of Béchar, Algeria.

The aerial parts (stems and leaves) of both species were collected in the Béchar region on 18 December 2023. *A. herba-alba* was collected from Oumm Sboaa, whereas *H. articulatum* was collected from Djorf Torba. After collection, the plant material was dried for 15 days at approximately 25°C in a cool place protected from direct light.

Plant identification was based on botanical literature, the national herbarium, an Ozenda identification key, and consultation with specialists in plant systematics and

physiology at the Faculty of Natural and Life Sciences, Tahri Mohammed University. The second plant is reported throughout as *Haloxylon articulatum*, the operational name used in the experimental records and the directly comparable literature cited here. No voucher specimen number was available in the experimental records used for this manuscript.

2.2. Hydrodistillation and co-hydrodistillation

Essential oils were obtained by hydrodistillation using Clevenger- and Dean–Stark-type apparatus. For single-plant extraction, 130 g of dried plant material were placed in a 2-L flask with 1 L of distilled water and heated to boiling for 3–4 h. Steam-borne volatiles were condensed and separated from the aqueous phase according to density.

In addition to the single-plant preparations, three co-hydrodistilled formulations were produced by combining the dried plant materials before distillation. The formulations were defined according to the actual plant masses used:

- **M1:** 50 g *A. herba-alba* + 50 g *H. articulatum*;
- **M2:** 40 g *A. herba-alba* + 60 g *H. articulatum*;
- **M3:** 60 g *A. herba-alba* + 40 g *H. articulatum*.

These formulations therefore represent products of co-hydrodistillation rather than post-extraction blending of independently produced oils. The overall experimental workflow is summarized in Figure 1.

After extraction, the recovered essential oils were transferred to sterile dark glass containers and stored at 4°C until physicochemical characterization and antifungal testing.

Table 1. Plant material and co-hydrodistillation design

Code	Preparation	Plant material used	Preparation type
AHA	<i>Artemisia herba-alba</i>	130 g dried aerial material for single-plant extraction	Hydrodistilled essential oil
HA	<i>Haloxylon articulatum</i>	130 g dried aerial material for single-plant extraction	Hydrodistilled essential oil
M1	AHA:HA 50:50	50 g AHA + 50 g HA	Co-hydrodistilled essential oil
M2	AHA:HA 40:60	40 g AHA + 60 g HA	Co-hydrodistilled essential oil
M3	AHA:HA 60:40	60 g AHA + 40 g HA	Co-hydrodistilled

Notes. AHA and HA were collected on 18 December 2023 in the Béchar region and dried for 15 days at approximately 25°C away from direct light.

Figure 1. Experimental workflow

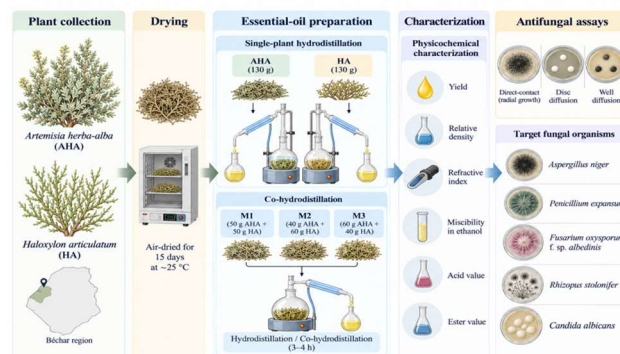


Figure 1. Experimental workflow for plant collection, drying, single-plant hydrodistillation, co-hydrodistillation, physicochemical characterization, and antifungal assays.

2.3. Essential-oil yield and physicochemical characterization

Essential-oil yield was expressed as a percentage of the dry plant material according to:

$$R_{HE} (\%) = (m_h / m_v) \times 100$$

where m_h is the mass of recovered essential oil (g) and m_v is the mass of plant material used for extraction (g).

Physicochemical characterization followed AFNOR methods. Relative density at 20°C was determined with a pycnometer (NF T 75-111). The mass of the empty pycnometer, the pycnometer filled with distilled water, and the pycnometer filled with essential oil were recorded, and relative density was calculated as:

$$D_{20} = (m_2 - m_0) / (m_1 - m_0)$$

where m_0 is the mass of the empty pycnometer, m_1 is the mass of the pycnometer filled with water, and m_2 is the mass of the pycnometer filled with essential oil.

The refractive index was measured at 20°C using a precision refractometer calibrated with distilled water to a reference value of 1.333 (NF T 75-112). Ethanol miscibility was evaluated at 20°C by progressively adding ethanol in 0.1-mL increments to 1 mL of essential oil until a clear solution was obtained (NF T 75-101).

The acid value was determined according to NF T 75-103. One gram of essential oil was mixed with 5 mL of 95% ethanol and phenolphthalein, then titrated with 0.1 N KOH. The acid value was calculated as:

$$IA = (5.61 \times V) / m$$

where V is the volume of KOH solution used (mL) and m is the mass of essential oil (g).

The ester value was determined according to NF T 75-104. One gram of essential oil was mixed with 25 mL of ethanolic KOH (0.5 N) and heated for 1 h. After cooling, distilled water and phenolphthalein were added, and the

excess KOH was titrated with 0.5 N HCl. A blank was performed under the same conditions. The ester value was calculated as:

$$IE = [28.05 \times (V_0 - V_1) / m] - IA$$

where V_0 is the HCl volume used for the blank, V_1 is the HCl volume used for the sample, m is the sample mass, and IA is the acid value.

2.4. Fungal strains and inoculum preparation

Five fungal organisms were evaluated: *Aspergillus niger*, *Penicillium expansum*, *Fusarium oxysporum* f. sp. *albedinis*, *Rhizopus stolonifer*, and *Candida albicans*. The first three filamentous fungi were obtained from the strain collection of Dr. M. Missoum (VERVSA, 2024). *C. albicans* ATCC 10231 was obtained from the teaching laboratory of the Faculty of Natural and Life Sciences, Tahri Mohammed University. *R. stolonifer* (RHS24) was locally isolated from contaminated bread and identified on the basis of macroscopic and microscopic characteristics.

Pure fungal cultures were maintained on acidified potato dextrose agar (PDA) slants at 4°C. Before each experiment, filamentous fungi were subcultured on PDA and incubated for 7 days at 25°C. Their identity was checked using colony morphology and microscopic examination. Microscopic identification used the adhesive-tape technique, with fungal material mounted in lactophenol and examined at $\times 10$, $\times 40$, and $\times 100$ magnification.

For the mold assays, inocula were adjusted to approximately 10^5 CFU/mL by spore counting with a Malassez counting chamber. Before antifungal testing, *C. albicans* was subcultured for 24 h at 30°C. A young pure culture was then suspended in 10 mL of distilled water, and the optical density was adjusted to 0.08–0.10, corresponding to approximately 10^5 CFU/mL.

2.5. Preparation of essential-oil test concentrations

Because the essential oils were not miscible with water, a 0.2% agar solution was used to facilitate dispersion in the culture medium. For the concentration series reported consistently in the experimental protocol, the essential oil preparation was first mixed with 3 mL of 0.2% agar solution and vortexed. One milliliter of this mixture was then added to 14 mL of PDA before pouring the medium into Petri dishes.

The principal concentrations evaluated were **0.007, 0.008, 0.012, and 0.014 $\mu\text{L/mL}$** . These concentrations constituted the principal test series used for the comparative analyses.

2.6. Direct-contact and radial-growth assays

For the direct-contact assay, essential-oil preparations were incorporated directly into PDA at the tested concentrations. Filamentous fungi were incubated at 25°C for 7 days and examined daily. For *C. albicans*, direct-contact growth was assessed after 48 h of incubation at 30°C.

For the radial-growth assay, the center of each PDA plate was point-inoculated. The plates were held at 4°C

for 4 h before incubation at 25°C for 7 days. Colony diameter was measured daily in millimeters and compared with an untreated control plate prepared without essential oil. Percentage inhibition of radial growth was calculated as:

$$I(\%) = 100 \times (dC - dE) / dC$$

where dC is the colony diameter in the untreated control and dE is the colony diameter in the essential-oil treatment.

For descriptive comparison, the lowest tested concentration producing complete absence of visible growth relative to the untreated control was recorded. This operational threshold is reported only where the available growth/no-growth record permitted direct identification; it should not be interpreted as a standardized CLSI/EUCAST MIC.

2.7. Agar-well diffusion assay

For the well-diffusion assay, inoculated agar plates were perforated with a sterile Pasteur pipette tip to produce wells approximately 6 mm in diameter. Each well received 30 μL of the tested essential-oil preparation at the stated concentration. The plates were maintained at 4°C for 4 h to allow diffusion before incubation.

Filamentous fungi were incubated at 25°C for 7 days with daily observation. *C. albicans* inhibition zones were measured after 24 h of incubation. Antifungal activity was evaluated from the diameter of the inhibition zone.

2.8. Disc-diffusion assay

For the disc-diffusion assay, the prepared fungal suspension was spread over the agar surface to obtain a confluent inoculum. After the plates had dried for several minutes, sterile 6-mm Whatman paper discs were impregnated with the essential-oil preparations at 0.007, 0.008, 0.012, or 0.014 $\mu\text{L/mL}$ and placed on the inoculated medium.

The plates were kept at room temperature for 1 h to allow diffusion and were then incubated at 25°C for 7 days for filamentous fungi. The yeast assay was read after 24 h. After incubation, inhibition-zone diameters were measured in millimeters, including the 6-mm disc diameter.

2.9. Data handling and reporting boundaries

The experimental design did not include systematic independent replication or inferential statistical analysis. Assays were repeated only when contamination occurred. Accordingly, the present article reports the observations descriptively and does not introduce ANOVA, t tests, p values, confidence intervals, or unsupported measures of dispersion.

An untreated culture served as the assay control. No reference antifungal drug or fungicide was included as a positive comparator. Consequently, the study is interpreted as an exploratory in vitro comparison of the tested plant preparations rather than as a formal efficacy or equivalence study.

The co-hydrodistilled formulations are evaluated as different plant-ratio preparations. Because no checkerboard, fractional inhibitory concentration index, isobolographic, or equivalent interaction analysis was performed, the study does not use the term **synergy** as a demonstrated experimental outcome.

3. Results

3.1. Essential-oil yield and physicochemical characteristics

The two single-plant preparations differed markedly in extraction yield. *Artemisia herba-alba* produced an essential-oil yield of 12.38%, whereas *Haloxylon articulatum* yielded 1.30% (Table 2). The co-hydrodistilled preparations also yielded essential oil; however, exact M1–M3 yield values were not retained in the quantitative comparison because the underlying records were internally inconsistent.

The physicochemical profiles also differed among the individual and co-hydrodistilled preparations. Relative density ranged from 0.710 for AHA to 0.918 for HA, whereas refractive index ranged from 1.331 for M1 to 1.455 for M3. The preparations also differed in ethanol miscibility, acid value, and ester value (Table 2). The M1 acid value was not retained numerically because two conflicting values were present in the source records.

Table 2. Extraction yield and physicochemical characteristics of single-plant and co-hydrodistilled preparations

Preparation	Yield (%)	Relative density	Refractive index	Ethanol miscibility (V:V)	Acid value (mg KOH/g)	Ester value (mg KOH/g)
AH	12.38	0.710	1.446	1:3	1.683	2.106
HA	1.30	0.918	1.331	1:1	1.230	1.490
M1	N [*]	0.823	1.331	1:1	N	2.930
M2	N [*]	0.891	1.331	1:1	0.673	1.250
M3	N [*]	0.778	1.455	1:2	1.122	0.521

Notes. AHA, *Artemisia herba-alba*; HA, *Haloxylon articulatum*; M1, 50:50 AHA:HA co-hydrodistillation; M2, 40:60 AHA:HA co-hydrodistillation; M3, 60:40 AHA:HA co-hydrodistillation; NR, not retained in the validated quantitative dataset.

* Exact M1–M3 yield values were not retained because the available primary records were internally inconsistent.

† The M1 acid value was not retained because two conflicting values were present in the available primary record.

3.2. Individual antifungal activity of *Artemisia herba-alba* and *Haloxylon articulatum*

AHA showed the broader individual-preparation antifungal profile in the direct-contact assays. Visible growth of *Fusarium oxysporum* f. sp. *albedinis* and *Rhizopus stolonifer* was completely inhibited from the lowest principal tested concentration of 0.007 μ L/mL and remained absent across the higher tested concentrations. *Candida albicans* ATCC 10231 similarly showed complete visible inhibition from 0.007 μ L/mL, after weaker growth responses at 0.0003 and 0.003 μ L/mL. By contrast, *Aspergillus niger* remained visibly growing throughout the 0.007–0.014 μ L/mL direct-contact series (Table 3).

The radial-growth records were consistent with a species-dependent response. Strong inhibition was reported for *F. oxysporum*, *P. expansum*, and *R. stolonifer*, whereas the response of *A. niger* was less complete. The daily radial-growth records indicated substantial variation across species and concentrations, although not every percentage could be interpreted unambiguously from the available notation.

HA showed a comparatively limited individual-preparation response. At 0.012 μ L/mL, the recorded radial-growth effects against the tested molds were weak or incomplete, and *C. albicans* still showed visible growth despite a reduction relative to the untreated control (Table 3). Accordingly, complete visible inhibition was not reached for *C. albicans* at the single HA concentration documented in the growth/no-growth table.

Diffusion assays further demonstrated organism-specific patterns for AHA. In the disc-diffusion assay, inhibition-zone diameters increased from 1 to 5 mm for *A. niger* and from 0 to 9 mm for *P. expansum* across the listed concentration series. In contrast, the recorded *C. albicans* disc-diffusion zones decreased from 15 to 6 mm as the listed concentration increased. This counter-directional pattern was retained as reported rather than corrected editorially. In the well-diffusion assay, inhibition zones generally increased with concentration for the five tested organisms, reaching 11 mm for *A. niger*, 8 mm for *F. oxysporum*, 16 mm for *P. expansum*, 9 mm for *R. stolonifer*, and 27 mm for *C. albicans* at 0.014 μ L/mL (Table 4).

3.3. Antifungal activity of the co-hydrodistilled formulations

The three co-hydrodistilled preparations showed different antifungal response profiles, indicating that activity varied with the plant ratio used during co-hydrodistillation.

For M1, *F. oxysporum* f. sp. *albedinis* showed complete visible inhibition across the tested 0.007–0.014 μ L/mL direct-contact series. *C. albicans* was completely

inhibited from 0.007 $\mu\text{L/mL}$. *R. stolonifer* remained visibly growing at 0.007, 0.008, and 0.012 $\mu\text{L/mL}$ but showed complete inhibition at 0.014 $\mu\text{L/mL}$. In contrast, *A. niger* remained visibly growing throughout the direct-contact concentration series (Table 3).

The diffusion assays for M1 showed a different pattern for *A. niger*. In disc diffusion, no inhibition zone was recorded at 0.007 $\mu\text{L/mL}$, while zones of 3, 8, and 8 mm were recorded at 0.008, 0.012, and 0.014 $\mu\text{L/mL}$, respectively. For *C. albicans*, disc-diffusion zones increased from 5 to 15 mm across the same concentration series. In the well-diffusion assay, *A. niger* zones increased from 0 to 8 mm, *R. stolonifer* from 0 to 10 mm, and *C. albicans* from 10 to 17 mm (Table 4).

For M2, complete or near-complete inhibition was reported mainly at the upper part of the tested concentration range. The recorded inhibition thresholds were approximately 0.014 $\mu\text{L/mL}$ for *A. niger* and *R. stolonifer* and approximately 0.012 $\mu\text{L/mL}$ for *F. oxysporum*. *P. expansum* was reported as inhibited from approximately 0.008 $\mu\text{L/mL}$. For *C. albicans*, visible growth was present at 0.012 $\mu\text{L/mL}$ but absent at 0.014 $\mu\text{L/mL}$, giving a lowest observed complete-inhibition threshold of 0.014 $\mu\text{L/mL}$ within the reported test range (Table 3).

M3 produced a distinct profile. *C. albicans* showed visible growth at 0.007 $\mu\text{L/mL}$ but complete visible inhibition from 0.008 $\mu\text{L/mL}$ upward. Recorded inhibition thresholds were approximately 0.012 $\mu\text{L/mL}$ for *A. niger* and *R. stolonifer*, whereas *F. oxysporum* and *P. expansum* showed complete inhibition throughout the tested series (Table 3). In the corresponding diffusion table, the recorded zones at 0.014 $\mu\text{L/mL}$ were 10 mm for *A. niger*, 8 mm for *F. oxysporum*, 2 mm for *P. expansum*, and 12 mm for *C. albicans* (Table 4).

Across M1–M3, no single co-hydrodistilled preparation displayed a uniformly superior response against all tested organisms. Instead, the observed activity depended on formulation, fungal species, concentration, and assay format. A comparative visual summary of the lowest tested concentrations producing complete visible inhibition is provided in Figure 2.

Table 3. Growth-inhibition profile and lowest tested concentrations producing complete visible inhibition

Preparation	Organism	Tested concentration range ($\mu\text{L/mL}$)	Lowest tested concentration with complete visible inhibition ($\mu\text{L/mL}$)	Evidence status	Main observation
AHA	<i>Aspergillus niger</i>	0.007–0.014	Not reached	Direct growth /no-growth record	Visible growth persisted in the direct-contact table
AHA	<i>Fusarium oxysporum</i> f. sp. <i>albedinis</i>	0.007–0.014	0.007	Direct growth /no-growth record	Complete visible inhibition across the tested range
AHA	<i>Rhizopus stolonifer</i>	0.007–0.014	0.007	Direct growth /no-growth record	Complete visible inhibition across the tested range
AHA	<i>Candida albicans</i> ATCC 10231	0.0003–0.014	0.007	Direct growth /no-growth record	Complete visible inhibition from 0.007
HA	<i>Candida albicans</i> ATCC 10231	0.012	Not reached	Direct growth /no-growth record	Growth was reduced but remained visible
M1	<i>Aspergillus niger</i>	0.007–0.014	Not reached	Direct growth /no-growth record	Growth persisted in direct contact; diffusion assays showed measurable zones
M1	<i>Fusarium oxysporum</i> f. sp. <i>albedinis</i>	0.007–0.014	0.007	Direct growth /no-growth record	Complete visible inhibition across the tested range
M1	<i>Rhizopus stolonifer</i>	0.007–0.014	0.014	Direct growth /no-growth record	Complete visible inhibition only at the

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					highest tested concentration					0.012																																																
M1	<i>Candida albicans</i> ATCC 10231	0.0003–0.014	0.007	Direct growth /no-growth record	Complete visible inhibition from 0.007	M3	<i>Fusarium oxysporum</i> f. sp. <i>albedinis</i>	0.007–0.014	≤0.007	Direct growth /no-growth record	Total inhibition reported across the stated range																																															
M2	<i>Aspergillus niger</i>	0.008–0.014	≈0.014	Narrative-supported approximate	Complete inhibition reported at the upper tested concentration	M3	<i>Penicillium expansum</i>	0.007–0.014	≤0.007	Direct growth /no-growth record	Total inhibition reported across the stated range																																															
M2	<i>Fusarium oxysporum</i> f. sp. <i>albedinis</i>	0.008–0.014	≈0.012	Narrative-supported approximate	Inhibition reported from approximately 0.012	M3	<i>Rhizopus stolonifer</i>	0.007–0.014	≈0.012	Narrative-supported approximate	Inhibition reported from approximately 0.012																																															
M2	<i>Penicillium expansum</i>	0.008–0.014	≈0.008	Narrative-supported approximate	Inhibition reported from approximately 0.008	M3	<i>Candida albicans</i> ATCC 10231	0.007–0.014	0.008	Direct growth /no-growth record	Complete visible inhibition from 0.008																																															
M2	<i>Rhizopus stolonifer</i>	0.008–0.014	≈0.014	Narrative-supported approximate	Inhibition reported at the upper tested concentration	Notes. Thresholds represent the lowest tested concentrations at which complete visible inhibition was recorded. “Not reached” refers only to the tested concentration range in the relevant assay. The symbol “≈” identifies approximate thresholds supported by narrative source records rather than a complete organism-specific growth/no-growth matrix. These values are descriptive and are not presented as standardized CLSI/EUCAST MIC estimates. Figure 2. Lowest tested concentrations producing complete visible inhibition (μL/mL) <table><tr><th>Preparation</th><th></th><th></th><th></th><th></th><th></th></tr><tr><td>AHA</td><td>Not reached</td><td>NR</td><td>0.007</td><td>0.007</td><td>0.007</td></tr><tr><td>HA</td><td>NR</td><td>NR</td><td>NR</td><td>NR</td><td>Not reached at 0.012</td></tr><tr><td>M1 (50:50)</td><td>Not reached</td><td>NR</td><td>0.007</td><td>0.014</td><td>0.007</td></tr><tr><td>M2 (40:60)</td><td>≈0.014</td><td>≈0.008</td><td>≈0.012</td><td>≈0.014</td><td>0.014</td></tr><tr><td>M3 (60:40)</td><td>≈0.012</td><td>≤0.007</td><td>≤0.007</td><td>≈0.012</td><td>0.008</td></tr></table> Color key (lower threshold = stronger observed activity) <table><tr><td>≤ 0.007 (strongest)</td><td>> 0.007 – 0.008</td><td>> 0.008 – 0.014</td><td>> 0.014</td><td>Not reached / NR (no complete inhibition)</td></tr></table> Abbreviations and symbols <table><tr><td>NR</td><td>= not reported</td></tr><tr><td>≈</td><td>= approximate narrative-supported threshold</td></tr><tr><td>≤</td><td>= less than or equal to the stated value</td></tr></table> Values are descriptive and are not standardized CLSI/EUCAST MIC estimates. Unit: μL/mL.						Preparation						AHA	Not reached	NR	0.007	0.007	0.007	HA	NR	NR	NR	NR	Not reached at 0.012	M1 (50:50)	Not reached	NR	0.007	0.014	0.007	M2 (40:60)	≈0.014	≈0.008	≈0.012	≈0.014	0.014	M3 (60:40)	≈0.012	≤0.007	≤0.007	≈0.012	0.008	≤ 0.007 (strongest)	> 0.007 – 0.008	> 0.008 – 0.014	> 0.014	Not reached / NR (no complete inhibition)	NR	= not reported	≈	= approximate narrative-supported threshold	≤	= less than or equal to the stated value
Preparation																																																										
AHA	Not reached	NR	0.007	0.007	0.007																																																					
HA	NR	NR	NR	NR	Not reached at 0.012																																																					
M1 (50:50)	Not reached	NR	0.007	0.014	0.007																																																					
M2 (40:60)	≈0.014	≈0.008	≈0.012	≈0.014	0.014																																																					
M3 (60:40)	≈0.012	≤0.007	≤0.007	≈0.012	0.008																																																					
≤ 0.007 (strongest)	> 0.007 – 0.008	> 0.008 – 0.014	> 0.014	Not reached / NR (no complete inhibition)																																																						
NR	= not reported																																																									
≈	= approximate narrative-supported threshold																																																									
≤	= less than or equal to the stated value																																																									
M2	<i>Candida albicans</i> ATCC 10231	0.012–0.014	0.014	Direct growth /no-growth record	Complete visible inhibition at 0.014																																																					
M3	<i>Aspergillus niger</i>	0.007–0.014	≈0.012	Narrative-supported approximate	Inhibition reported from approximately																																																					

Figure 2. Heatmap-style summary of the lowest tested concentrations producing complete visible inhibition across individual and co-hydrodistilled preparations. Values are descriptive and are not standardized CLSI/EUCAST MIC estimates.

3.4. Assay-dependent and species-specific response patterns

The clearest cross-assay difference concerned *A. niger*. In the direct-contact tables, visible growth persisted across the tested concentration series for AHA and M1. Nevertheless, measurable inhibition zones were recorded for the same organism in diffusion-based assays. With AHA, the well-diffusion zone increased from 3 mm at 0.007 $\mu\text{L/mL}$ to 11 mm at 0.014 $\mu\text{L/mL}$. With M1, measurable inhibition zones also appeared at the higher tested concentrations in both disc- and well-diffusion assays (Table 4). This assay-dependent pattern is illustrated in Figure 3.

This pattern was not restricted to *A. niger*. The magnitude and direction of the recorded responses varied across organisms and assay formats. *C. albicans*, for example, showed complete visible inhibition in direct-contact tests at relatively low concentrations for AHA and M1, while its diffusion-zone pattern differed by assay. Likewise, *P. expansum* displayed stronger inhibition in some well-diffusion records than in the corresponding disc-diffusion measurements.

Overall, the experimental evidence therefore indicated a heterogeneous antifungal response rather than a single rank order that applied across all organisms and methods. The most consistent findings were the broader individual-preparation activity of AHA, the comparatively limited individual-preparation activity of HA, formulation-dependent responses among M1–M3, and clear assay dependence in the apparent susceptibility of selected fungi.

Table 4. Observed inhibition-zone diameters in diffusion assays

Panel A. AHA — disc diffusion				
Organism	0.007	0.008	0.012	0.014
<i>A. niger</i>	1	2	4	5
<i>P. expansum</i>	0	0	5	9
<i>C. albicans</i>	15	10	7	6

Panel B. AHA — well diffusion				
Organism	0.007	0.008	0.012	0.014
<i>A. niger</i>	3	5	7	11
<i>F. oxysporum</i>	0	2	4	8
<i>P. expansum</i>	7	9	14	16
<i>R. stolonifer</i>	0	0	5	9
<i>C. albicans</i>	4	7	10	27

Panel C. M1 — disc diffusion				
Organism	0.007	0.008	0.012	0.014
<i>A. niger</i>	0	3	8	8
<i>C. albicans</i>	5	8	10	15

Panel D. M1 — well diffusion				
Organism	0.007	0.008	0.012	0.014
<i>A. niger</i>	0	2	5	8
<i>R. stolonifer</i>	0	0	7	10
<i>C. albicans</i>	10	15	17	17

Panel E. M3 — diffusion table 16				
Organism	0.007	0.008	0.012	0.014
<i>A. niger</i>	1	5	8	10
<i>F. oxysporum</i>	2	5	8	8
<i>P. expansum</i>	0	0	0	2
<i>C. albicans</i>	4	6	8	12

Unit: mm.

Reporting boundary: an additional M3 diffusion dataset was not assigned to a specific assay because its original assay label was ambiguous; it is therefore excluded from the main quantitative comparison.

Figure 3. Assay-dependent response of *Aspergillus niger*

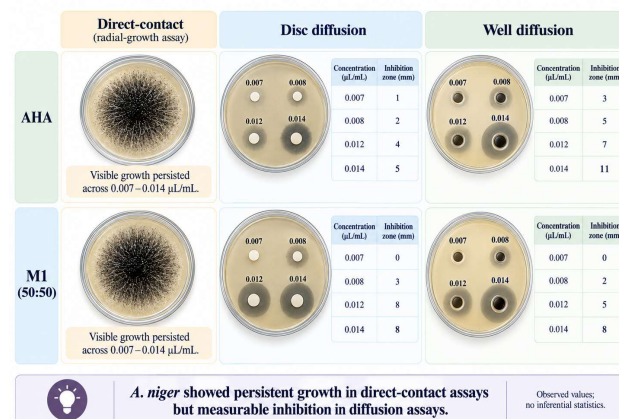


Figure 3. Assay-dependent response of *Aspergillus niger* in direct-contact, disc-diffusion, and well-diffusion assays for AHA and M1. Diffusion values are observed inhibition-zone diameters; no inferential statistics are implied.

4. Discussion

The present study compared the antifungal activity of essential oils obtained from *Artemisia herba-alba* and *Haloxylon articulatum*, both individually and through co-hydrodistillation at different plant ratios. Four patterns are central to the interpretation of the results. First, *A. herba-alba* showed the broadest individual-preparation antifungal profile under the tested conditions. Second, *H. articulatum* displayed comparatively limited individual-

preparation activity. Third, changing the relative proportion of the two plants during co-hydrodistillation altered the observed antifungal profile, but no single formulation was uniformly superior across all tested organisms. Fourth, apparent susceptibility depended on the assay format, with *Aspergillus niger* providing the clearest example of divergence between direct-contact and diffusion-based measurements. The results therefore favor a formulation-, organism-, and assay-dependent interpretation over a single overall potency ranking.

4.1. Broad antifungal activity of *Artemisia herba-alba*

The broad individual-preparation activity of *A. herba-alba* is consistent with reports of antifungal effects against yeasts, molds, dermatophytes, and phytopathogenic fungi. Abu-Darwish et al. (2015) reported antifungal activity against several fungal groups and substantial inhibition of *Candida albicans* germ-tube formation. Houti et al. (2023) documented activity against molds including *A. niger* and *Penicillium expansum*, while Lazrega et al. (2025) recently reported strong inhibition of *Fusarium oxysporum* f. sp. *ciceri* by Algerian *A. herba-alba* essential oil. Earlier Tunisian work also showed broad antimicrobial activity among chemically distinct *A. herba-alba* oil types (Mighri et al., 2010). These findings are consistent with an established body of evidence on *A. herba-alba* essential oil and extend it to the strains and assay conditions examined here.

The response was nevertheless not uniform across fungal taxa. In the direct-contact assay, *F. oxysporum* f. sp. *albedinis*, *Rhizopus stolonifer*, and *C. albicans* showed complete visible inhibition at relatively low tested concentrations, whereas *A. niger* continued to grow across the same principal concentration range. Such heterogeneity is consistent with broader essential-oil literature. Powers et al. (2019), in a comparative analysis of 82 essential oils, found marked differences in susceptibility among *A. niger*, *C. albicans*, and *Cryptococcus neoformans*. Essential-oil studies against postharvest fungi likewise show that *Penicillium*, *Aspergillus*, and *Rhizopus* species can differ substantially in the concentration required for inhibition (Xing et al., 2010). The organism-level variation argues against assigning a single global “active” or “inactive” label to an essential oil.

Differences between the current findings and published potency estimates should also be interpreted cautiously because the chemical composition of *A. herba-alba* is known to vary with geography, harvest period, and plant material. Algerian and Jordanian oils have shown different dominant constituents and yields (Dob & Benabdelkader, 2006; Hudaib & Aburjai, 2006), while Mighri et al. (2010) identified several chemically distinct oil types from plants cultivated in the Tunisian arid zone. Ouguirti et al. (2021) further documented a chemically characterized oil from southwestern Algeria, and

Elwardani et al. (2024) demonstrated seasonal changes in yield, composition, and antifungal performance. Houti et al. (2023) similarly reported harvest-related variability. Such evidence makes compositional variation a credible explanation for differences among studies, but it cannot be tested directly here because the present oils were not characterized by GC–MS. The current antifungal activity should therefore not be attributed to thujone, camphor, 1,8-cineole, or any other individual constituent without chemical evidence from the tested samples.

The activity observed against *F. oxysporum* f. sp. *albedinis* is also relevant in the Algerian phytopathological context. Boukhobza et al. (2021) showed that an Algerian rosemary essential oil could inhibit *F. oxysporum* f. sp. *albedinis*, while Lazrega et al. (2025) demonstrated that *A. herba-alba* oil can strongly inhibit another form specialis of *F. oxysporum*. These studies do not provide direct quantitative comparators for the present experiment because the oils, strains, and methods differ, but they support continued evaluation of aromatic-plant essential oils against *Fusarium* pathogens under regionally relevant conditions.

4.2. Limited individual-preparation activity of *Haloxylon articulatum*

In contrast to *A. herba-alba*, the *H. articulatum* preparation showed only limited standalone antifungal activity under the conditions evaluated. The recorded response of *C. albicans* at 0.012 $\mu\text{L/mL}$ illustrates this distinction: growth was reduced relative to the untreated control but remained visible, so complete inhibition was not reached at the documented concentration. The mold assays similarly indicated weak or incomplete responses.

This pattern is broadly consistent with related work reported under *Hammada scoparia*. Drorioche et al. (2020) found limited antifungal performance in their antimicrobial evaluation despite evidence of other biological activities. However, the literature does not justify a broader conclusion that *H. articulatum* lacks antifungal potential. Lakhdari et al. (2024) reported substantial antifungal activity of a methanolic *H. articulatum* extract against selected phytopathogenic fungi, including strong effects against *Thielaviopsis paradoxa* and *Alternaria* sp. The difference in extraction system is critical because a methanolic extract and a hydrodistilled essential oil capture different fractions of plant chemistry and are not directly comparable.

Together, these studies define an important boundary for interpreting the present result. The limited activity recorded for HA should be interpreted specifically as the behavior of the tested essential-oil preparation under the present concentrations and assay conditions. It does not establish low antifungal potential for the plant as a whole. Differences in extraction method, fungal target, geographical origin, plant chemistry, and assay conditions remain credible explanations for the variation across published studies. This is especially relevant for *H.*

articulatum, for which the antifungal literature is much thinner than for *A. herba-alba* and often spans extracts of different polarity rather than directly comparable essential oils.

4.3. Plant-ratio dependence of the co-hydrodistilled formulations

The three co-hydrodistilled preparations did not reproduce one common antifungal profile. M1 showed strong direct-contact activity against *F. oxysporum* and *C. albicans*, while *R. stolonifer* required the highest tested concentration for complete visible inhibition and *A. niger* continued to grow in the direct-contact series. M2 generally showed complete inhibition only toward the upper part of the tested range, whereas M3 displayed a different pattern, including complete inhibition of *C. albicans* from 0.008 $\mu\text{L/mL}$ and strong responses against *F. oxysporum* and *P. expansum*. Collectively, these observations indicate that different plant ratios during co-hydrodistillation were associated with different observed biological profiles.

This finding should be distinguished from the more common design in which independently extracted essential oils are physically mixed after distillation. In a co-distillation design, the two plant materials are distilled together, and the resulting product may not be chemically equivalent to a post-extraction blend. Nebić et al. (2023) addressed this distinction directly by comparing an essential oil obtained through co-distillation of *Cymbopogon citratus* and *Eucalyptus camaldulensis* with a mixture prepared from the two separately extracted oils. Their co-distilled preparation differed in chemical composition and showed antifungal activity against several phytopathogenic fungi. In another two-plant system, Nebić et al. (2024) likewise showed that co-distillation generated a volatile profile distinct from those of the parent oils and from their simple mixture, although the biological endpoint in that study was insecticidal rather than antifungal.

These studies provide a plausible process-level explanation for the differing M1–M3 profiles. Changing the relative amount of each plant during simultaneous distillation could alter the relative recovery of volatile constituents and, consequently, the biological profile of the resulting oil. Nevertheless, this mechanism remains literature-supported rather than demonstrated in the present study because M1–M3 were not chemically profiled. The observed differences can therefore support a claim of **formulation-dependent activity**, but not a claim that co-distillation chemically enriched or depleted any particular constituent.

The same boundary applies to the term “synergy.” Essential-oil combinations can display synergistic, additive, indifferent, or antagonistic effects, but formal interaction claims require an appropriate quantitative framework. Bassolé and Juliani (2012) emphasized that antimicrobial interactions among essential oils or their

components should be evaluated using specific combination methods rather than inferred from an apparently larger inhibition response. No checkerboard assay, fractional inhibitory concentration index, isobolographic analysis, or equivalent interaction design was performed here. Consequently, the present results support the conclusion that co-hydrodistillation altered the antifungal profile, but they do not demonstrate synergism between *A. herba-alba* and *H. articulatum*.

4.4. Assay-dependent antifungal response

The differences observed among direct-contact, disc-diffusion, and well-diffusion assays are a central methodological finding of the study. *A. niger* was particularly informative: visible growth persisted in the direct-contact tables for AHA and M1, whereas measurable inhibition zones were obtained in diffusion assays. This discrepancy does not identify one assay as inherently “correct”; rather, it indicates that apparent activity depends partly on how exposure is generated and measured.

This interpretation is strongly supported by methodological literature. Donaldson et al. (2005) reported that dilution results were only weakly correlated with agar-diffusion results and highlighted volatility and poor aqueous solubility as important constraints. Tullio et al. (2007) likewise found that antifungal activity differed between broth microdilution and vapor contact, with vapor-phase inhibition often exceeding that observed in liquid exposure. Cavanagh (2007) emphasized that inconsistencies in volatile-phase testing and reporting can hinder comparison across studies. More recently, Rashed et al. (2021) reviewed the limitations of disc, well, and agar-based methods for essential oils, noting that components differ in aqueous dispersion, volatilization, and agar penetration. These observations are directly relevant to the present data because the direct-contact method distributes the preparation within the medium, whereas disc and well methods depend on localized diffusion through agar.

The *A. niger* pattern also has a close empirical analogue in the work of Bouddine et al. (2012), who compared several essential oils against *A. niger* using broth dilution, disc diffusion, and a micro-atmosphere method. Apparent antifungal efficacy changed markedly across methods. Although their oils and experimental conditions differed from those used here, the study demonstrates that assay format can materially change the apparent susceptibility of *A. niger* to volatile plant products. Similar method dependence is also consistent with broader reviews of antifungal and antimycotoxigenic essential-oil research, in which concentration, matrix, and exposure mode are repeatedly identified as major sources of variability (Mutlu-Ingok et al., 2020).

Several properties of essential oils can plausibly contribute to such differences, including hydrophobicity, limited miscibility in aqueous media, agar diffusion

behavior, volatility, and interactions with the culture matrix. These explanations are supported by methodological literature but were not isolated experimentally here. The safest interpretation is therefore that the assays captured different dimensions of exposure rather than interchangeable estimates of one underlying potency value. This also explains why inhibition-zone diameter should not be treated as directly equivalent to a MIC derived from contact or dilution assays.

4.5. Scientific implications and study boundaries

The principal contribution is not the identification of a universally superior essential oil or co-distilled formulation. Instead, the evidence shows that antifungal performance depends jointly on botanical source, co-hydrodistilled formulation, fungal species, concentration, and assay format. This is a more defensible interpretation than reducing the dataset to a single ranking. In particular, the results support three practical lessons for future experimental work: single-plant activity should be established before evaluating combined preparations; co-hydrodistilled formulations should be treated as distinct products rather than assumed to reproduce simple oil blends; and more than one assay format may be needed when evaluating volatile, poorly water-miscible antifungal agents.

These conclusions must be interpreted within several methodological limitations. First, the original experimental design did not include systematic biological or technical replication, and no inferential statistical analysis was performed. The study therefore supports descriptive comparisons but not claims of statistical superiority. Second, no reference antifungal compound was included as a positive comparator, preventing direct benchmarking against a standardized drug or fungicide. Third, no GC–MS analysis was performed on the tested oils or co-hydrodistilled formulations, so the chemical basis of the observed activity remains unresolved. Fourth, *R. stolonifer* was identified morphologically and microscopically rather than molecularly. Finally, some source records contained internally inconsistent concentration-related or physicochemical entries. Values that could not be reconciled from direct experimental evidence were excluded or explicitly qualified.

These limitations constrain, rather than negate, the observed in vitro patterns. The current evidence is best viewed as exploratory comparative evidence showing that *A. herba-alba* showed the broadest individual-preparation antifungal profile, that *H. articulatum* alone was less active under the present conditions, that the observed response profile differed across co-hydrodistillation ratios, and that susceptibility estimates were assay-dependent. A stronger confirmatory design would require predefined independent replication, standardized positive controls, chemical characterization of each oil and co-hydrodistilled preparation, and a formal interaction framework such as checkerboard/FICI analysis if synergy

is to be tested explicitly. Such a design would allow future work to distinguish whether the formulation-dependent effects observed here arise from altered volatile composition, true biological interactions among constituents, or methodological differences in compound delivery.

5. Conclusion

This study examined the in vitro antifungal activity of essential oils obtained from *Artemisia herba-alba* and *Haloxylon articulatum*, both individually and after co-hydrodistillation at different plant ratios. The findings show that antifungal performance cannot be reduced to a single potency ranking, because the observed response varied according to botanical source, co-hydrodistilled formulation, fungal species, concentration, and assay format. Among the two single-plant preparations, *A. herba-alba* displayed the broadest antifungal profile under the tested conditions, whereas *H. articulatum* showed comparatively limited individual-preparation activity. The three co-hydrodistilled preparations also produced distinct response patterns, indicating that changes in the relative proportion of the two plants during simultaneous distillation were associated with changes in the resulting antifungal profile. These differences, however, should not be interpreted as evidence of synergism because no formal interaction analysis was performed.

The results further demonstrate that apparent fungal susceptibility depended on the assay used. This was particularly evident for *Aspergillus niger*, which remained visibly growing under some direct-contact conditions while measurable inhibition zones were recorded in diffusion-based assays. The study therefore supports an assay-specific interpretation of essential-oil antifungal activity and highlights the limitations of reducing biological performance to a single measurement obtained from one experimental format. From this perspective, the main contribution of the study lies in providing a comparative framework in which the two single-plant essential oils and three co-hydrodistilled formulations prepared at three plant ratios were evaluated against several fungal organisms using different antifungal assays. By preserving formulation-, species-, and assay-dependent heterogeneity, the study provides a more precise basis for interpreting the antifungal potential of these plant preparations.

These findings should nevertheless be interpreted within the limitations of the experimental design. The study did not include systematic independent replication or inferential statistical analysis, and the results therefore support descriptive comparisons rather than claims of statistically significant differences. No reference antifungal drug or fungicide was included as a positive comparator, which prevents direct benchmarking of the tested preparations against a standardized treatment. In addition, the essential oils and co-hydrodistilled formulations were not characterized by GC–MS, so the

chemical basis of the observed differences remains unresolved and the activity cannot be attributed to specific volatile constituents. *Rhizopus stolonifer* was identified by macroscopic and microscopic characteristics rather than molecular methods, and some source records contained internal inconsistencies that could not be resolved from the available primary evidence; such values were therefore excluded or conservatively qualified.

Future work should address these limitations through predefined independent replication, standardized positive antifungal controls, and chemical characterization of each individual oil and co-hydrodistilled formulation. GC–MS profiling would make it possible to determine whether changes in plant ratio during co-hydrodistillation are associated with changes in volatile composition and whether such changes correspond to differences in antifungal response. If the objective is to test synergy explicitly, future studies should incorporate validated interaction designs such as checkerboard assays and fractional inhibitory concentration indices rather than inferring synergy from stronger inhibition alone. Extending the analysis to multiple isolates of the same fungal species and combining direct-contact, dilution, disc-diffusion, and well-diffusion methods within the same replicated design would also help distinguish genuine biological differences from effects related to dispersion, volatility, or agar diffusion. Overall, the present study provides exploratory comparative evidence that justifies more controlled and chemically characterized investigations of *A. herba-alba*, *H. articulatum*, and their co-hydrodistilled formulations as potential sources of antifungal activity.

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