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IN VITRO ANTIFUNGAL EFFECT OF *ROSMARINUS OFFICINALIS* L. ESSENTIAL OILS AGAINST *FUSARIUM* SP.

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Abstract

The in vitro antifungal activity of essential oils extracted from Rosmarinus officinalis L. was evaluated against Fusarium graminearum, a pathogen isolated from infected maize (Zea mays) plants. Three essential oil samples were tested: one obtained by laboratory distillation and two commercial products. Antifungal activity was assessed using two methods: incorporation of the oils into the culture medium to quantify mycelial growth inhibition, and application of filter paper discs on PDA medium to observe localized fungal inhibition. All three essential oils exhibited inhibitory effects on the growth of Fusarium graminearum, with the laboratory-extracted oil showing the highest efficacy. The incorporation method provided quantitative data on mycelial growth reduction, while the disc method served as a qualitative complementary approach. These results highlight the potential of R. officinalis essential oil, particularly when freshly extracted, as a natural antifungal agent against Fusarium graminearum affecting maize crops.

Key words*: Rosmarinus officinalis; Fusarium spp; maize; antifungal activity; in vitro.*

INTRODUCTION

Essential oils (EO) are volatile extracts primarily obtained from aromatic and medicinal plants. They serve as a rich source of biologically active compounds with antibacterial, insecticidal, fungicidal, nematicidal, herbicidal, antioxidant, and anti-inflammatory properties (Bakkali et al., 2008; Valerio et al., 2021). Historically, medicinal and aromatic plants (MAP) have been used to produce essential oils, mainly for flavoring and fragrance purposes, rooted in the tradition of using aromatic plants as culinary herbs and spices (Buckle, 2014). Among the plants with culinary and medicinal uses (Vâșcă-Zamfir et al., 2018), we mention the species *Rosmarinus officinalis* L. (Lamiaceae), also known for its antibacterial and antifungal properties. Over time, their applications have expanded into medicine, where they are now widely used in phytopharmaceuticals and aromatherapy (El Asbahani et al., 2015).

Beyond their physiological roles, these volatile compounds also perform essential ecological functions. They can act as internal signaling molecules, natural defense mechanisms against herbivores, or attractants for pollinators (Hedden et al., 2002). More recently, research has shifted toward their potential as biocontrol agents, employing low concentrations of EO to manage plant pests and pathogens (Isman, 2020; Andrés et al., 2012). This has positioned them as a promising alternative to synthetic pesticides, offering sustainable solutions for crop protection and pest control (Prabha et al., 2016). The increasing interest in EO applications has driven extensive academic and industrial research into their biological activities (Turek & Stintzing, 2012; Bassolé & Juliani, 2012). Biocontrol, a growing concept in pest management, has gained attention as part of integrated pest management (IPM) strategies (Barzman et al., 2015). The shift toward eco-friendly biopesticides is seen as a viable alternative to synthetic chemicals, reducing environmental contamination while maintaining agricultural productivity (Isman, 2019).

Numerous studies have demonstrated the biopesticidal potential of essential oils, particularly their antifungal, insecticidal, and nematicidal effects (Raveau et al., 2020; Arraiza et al., 2018). However, their direct use as

biopesticides presents several challenges, including phytotoxicity-long considered a major limitation in their development as insecticides or fungicides (Werrie et al., 2020) - their impact on food organoleptic properties, and the need for precise application rates to ensure efficacy (Turek & Stintzing, 2013).

Furthermore, their high volatility and susceptibility to degradation reduce their effectiveness when applied directly in the field (Turek & Stintzing, 2013). Although some commercial products based on essential oils-such as orange oil or essential oil blends-are already available on the market, further research is still needed to optimize their use in integrated biocontrol strategies and to expand their application to a wider range of pathogens and crops (Ravensberg, 2015).

The chemical composition of essential oils varies significantly across regions and even within the same geographical area due to differences in environmental conditions (El Asbahani et al., 2015; Turek & Stintzing, 2012), as well as depending on the phenophase and the parts of the plant (Badea et al., 2021). Addressing these variations and improving EO formulations will be crucial for their broader adoption in sustainable agriculture.

The phenological stage of the plant significantly influences EO yield, which differs among species (Tamokou et al., 2017). Typically, EOs from medicinal and aromatic plants are extracted during the flowering stage, prior to seed germination. However, this practice can hinder plant regeneration, making high-yield extraction challenging and underscoring the need for the domestication of medicinal and aromatic plants (MAPs) (Isman, 2019).

Unregulated harvesting of MAPs often leads to habitat loss, putting pressure on wild plant populations (Barzman et al., 2015). This issue is particularly critical in developing countries such as India, China, Nepal, Kenya, Tanzania, and Uganda, where the decline of medicinal plant species has accelerated (Bakkali et al., 2008). To meet the rising demand for standardized, high-quality raw materials in industrial markets, medicinal and aromatic plants have been systematically domesticated and cultivated (Isman, 2019). Additionally, many wild plants with human uses are now being cultivated as Crop Wild Relatives (CWR) to support conservation efforts (Valerio et al., 2021).

The aim of this study was to evaluate the in vitro antifungal activity of essential oils extracted from *Rosmarinus officinalis* L. against *Fusarium* spp., by comparing the efficacy of a laboratory-extracted oil with two commercial samples using two testing methods: incorporation into culture medium, which provided quantitative data on mycelial growth inhibition, and the filter paper disc method, which offered qualitative observations of fungal growth suppression.

MATERIALS AND METHODS

The biological material studied consisted of *Rosmarinus officinalis* L. plants grown in the medicinal and aromatic plant garden of the Faculty of Agriculture, specializing in Biology.

The essential oil from the rosemary samples was extracted using a Clevenger-type apparatus. The pre-prepared rosemary samples were distilled in water for three hours before the boiling process began. Approximately 400 g of fresh rosemary plant material (in the flowering phenophase) was used for the oil extraction, maintaining a 1:3 plant material-to-water ratio.

The antifungal activity of the essential oils from *Rosmarinus officinalis* L. was tested at the Research Center for the Study of Agro-Food Product Quality, within the University of Agronomic Sciences and Veterinary Medicine of Bucharest. The antifungal effects were compared to other commercially available essential oils, with the following experimental variants:

Pure, natural rosemary essential oil;

Commercial rosemary essential oil labeled as "S1";

Commercial rosemary essential oil labeled as "S2".

The microbiological material used for the experiment was *Fusarium* sp., isolated from *Zea mays* L., *Fusarium* spp. represents a complex of phytopathogens that colonize plant organs, particularly attacking seeds, causing rot, tracheomycosis, qualitative deterioration, yield reduction, and, in the case of *Fusarium graminearum*, mycotoxin contamination that affects the food chain (Cristea et al., 2024).

To evaluate the *in vitro* antifungal efficacy against Fusarium sp. isolated from corn cobs, two methods were used: the incorporation into culture medium (Food Poison Technique) to assess mycelial growth inhibition quantitatively, and a modified Kirby-Bauer disc diffusion method, using filter paper discs impregnated with essential oil at the tested concentrations. The inclusion method was applied at a concentration of 1%, chosen as a preliminary dose based on previous studies reporting antifungal effects of *Rosmarinus officinalis* essential oil. The essential oils were emulsified in 0.1% Tween 80 to enhance dispersion in the culture medium. The antifungal effectiveness was evaluated by measuring the radial growth of *Fusarium* sp. mycelium on treated plates compared to untreated controls. The percentage of mycelial growth inhibition was calculated using the formula: Inhibition (%) = C-T/C X 100.

Where C is the average colony diameter in the control, and T is the average diameter in the treated variant

The diffusion method with filter paper discs was applied, using a protocol adapted from the Kirby-Bauer method. Discs were impregnated with the same 1% essential oil solution used in the inclusion method, then placed on solidified PDA medium previously cooled to 45°C.

Fungal discs (7 mm diameter) were collected from the edge of actively growing Fusarium sp. cultures using a sterile cork borer. Petri dishes containing PDA medium were first inoculated centrally with a fungal disc, after which filter paper discs impregnated with 10 µL of rosemary essential oil (1% concentration) were placed at a distance of approximately 40 mm from the fungal inoculum. This distance was selected to allow clear observation of potential inhibition zones, based on modifications of standard disc diffusion protocols (Bauer et al., 1966; adapted for filamentous fungi).

In the experiments assessing the antifungal activity of rosemary essential oils, the fungal strain (Fusarium sp.) was incubated at 25 ± 1°C in darkness for 3, 6, and 9 days on PDA medium supplemented with a 1% essential oil solution emulsified in 0.1% Tween 80. The same amount of emulsifier was added to the control plates without essential oil. Fungal growth was monitored by measuring colony diameter with a graduated ruler, allowing for the assessment of the strain's sensitivity to the tested oils.

Petri dishes containing PDA medium without essential oil were used as control variants to evaluate the normal growth of the fungal strain in the absence of treatment (Figure 1).

The antifungal activity of rosemary essential oil was assessed by measuring the radial growth of *Fusarium* sp. colonies at 3, 6, and 9 days. The results, presented in Table 1, show a progressive increase in mycelial growth over time. Compared to the control, the treatment with rosemary essential oil resulted in an average inhibition of 7% after 9 day.

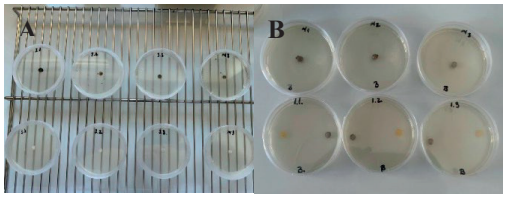

Figure 1. Method of Inclusion in Medium (A) and Diffusion Method with Filter Paper Discs (B) (original)

Table 1. Mycelial growth of *Fusarium* sp. on PDA medium (control), measured over time

Day	Replicate 1 (mm)	Replicate 2 (mm)	Replicate 3 (mm)	Mean Diameter (mm)	Growth Rate (mm/day)*
3	21.5	22.5	19.0	21.0	7.0
6	46.5	45.5	46.0	46.0	7.7
9	63.5	67.0	59.0	63.2	7.0

*Growth rate calculated as mean diameter / number of days.

RESULTS AND DISCUSSIONS

In the experiments testing the antifungal activity of rosemary essential oils, the studied strain was incubated under controlled conditions for 3, 6, and 9 days on PDA culture medium supplemented with a 1% volatile oil solution. The development of the strain was monitored,

and the diameter was measured using a graduated ruler, allowing for the evaluation of the pathogenic microorganisms' sensitivity level.
Petri dishes containing PDA medium without essential oil were prepared to observe the natural development of the pathogen (Figure 2).
The treatment with pure rosemary essential oil significantly inhibited the mycelial growth of *Fusarium* sp. at all observation intervals. Compared to the control, the inhibition rates were 61.9% after 3 days, 75.7% after 6 days, and 68.7% after 9 days (Table 2 and Table 3). These results confirm the strong antifungal potential of the tested oil.

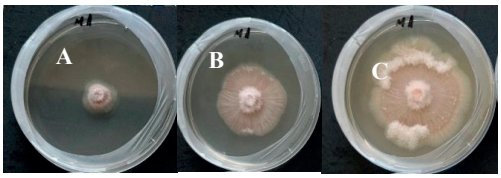

Figure 2. Evolution of the pathogen at 3 (A), 6 (B), and 9 (C) days on the control plates (sursă: original)

Table 2. Mycelial growth of *Fusarium* sp. on PDA medium supplemented with pure rosemary essential oil

Day	Replicate 1 (mm)	Replicate 2 (mm)	Replicate 3 (mm)	Mean Diameter (mm)
3	12.0	12.0	0.0	8.0
6	13.0	10.5	10.0	11.2
9	29.0	13.5	17.0	19.8

Table 3. Results

Day	Mean Control (mm)	Mean EO Treatment (mm)	Inhibition (%)
3	21.0	8.0	61.9
6	46.0	11.2	75.7
9	63.2	19.8	68.7

The commercial essential oil sample S1 also showed antifungal activity, particularly at the early stage of fungal development. At 3 days post-inoculation, the inhibition reached 78.6%, followed by lower values at 6 days (42.8%) and 9 days (27.5%), suggesting a reduced long-term effect compared to the pure oil treatment (Table 4 and Table 5).

Table 4. Mycelial growth of *Fusarium* sp. on PDA medium supplemented with commercial rosemary essential oil (S1)

Day	Replicate 1 (mm)	Replicate 2 (mm)	Replicate 3 (mm)	Mean Diameter (mm)
3	13.5	0.0	0.0	4.5
6	36.0	26.0	17.0	26.3
9	61.0	45.0	31.5	45.8

Tabel 5. Inhibition percentage vs. Control

Day	Mean Control (mm)	Mean S1 (mm)	Inhibition (%)
3	21.0	4.5	78.6
6	46.0	26.3	42.8
9	63.2	45.8	27.5

In repetitions 2 and 3, growth was slower, as the essential oil had inhibited pathogen development at day 3 (Figure 3).

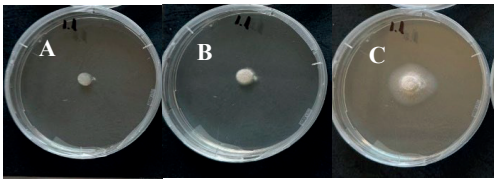

Figure 3. Evolution of the pathogen at 3 (A), 6 (B), and 9 (C) days on the plates enriched with pure essential oil (original)

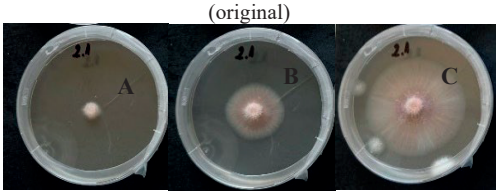

Figure 4. Pathogen development at 3 (A), 6 (B), and 9 (C) days on plates inoculated with the commercial essential oil "S1" (original)

The growth diameter recorded at 3 days (72 hours) post-inoculation with *Fusarium* spp. in the medium enriched with the commercial essential oil extract "S2" showed limited development, exhibiting a significant inhibitory effect on the plates in repetitions 2 and 3.

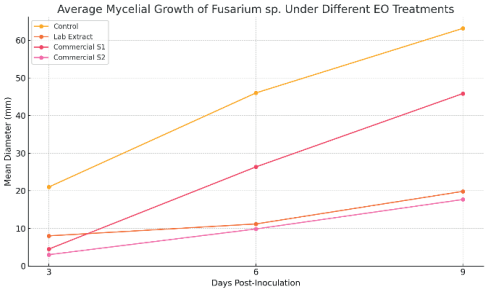

Figure 5. Comparative mycelial growth of Fusarium sp. on PDA medium supplemented with rosemary essential oils (lab-extracted and commercial samples) over 3, 6, and 9 days

Observations conducted at 6 days post-inoculation revealed growth diameters of 10 mm (replicate 1), 12 mm (replicate 2), and 7.5 mm (replicate 3), with a calculated mean of 9.83 mm. At 9 days, the recorded diameters were 15 mm, 19.5 mm, and 18.5 mm, respectively, resulting in a mean value of 17.67 mm. These data are summarized in Figure 5

Filter paper disc diffusion method

In the experiments assessing the antifungal activity of rosemary essential oils, the studied strains were incubated under controlled conditions for 3, 6, and 9 days on PDA culture medium. Petri dishes containing PDA were first inoculated with a spore suspension of *Fusarium* sp., after which sterile paper discs impregnated with 10 µL of a 1% essential oil solution (diluted in 0.5% Tween 80) were placed on the surface of the medium. The distance between the fungal inoculation point and the essential oil - impregnated disc was approximately 40 mm. The method was adapted to assess the radial diffusion of the essential oil and its potential inhibitory effect on mycelial growth.. Their development was monitored, and the growth diameter was measured using a graduated ruler, allowing for the evaluation of the pathogenic microorganisms' sensitivity. Petri dishes containing PDA medium without essential oil discs were used as control to assess the normal growth rate of the pathogen.

The fungal growth diameter was measured at 3, 6, and 9 days post-inoculation, following the same methodology applied to the plates treated with essential oil discs. Three biological replicates were performed for each experimental time point. At 3 days, the average diameters were 12.5 mm for replicate 1, 13 mm for replicate 2, and 13.5 mm for replicate 3.

On day 6, the average diameters recorded were 36.5 mm for both replicate 1 and 2, and 43 mm for replicate 3. At 9 days, the growth diameters were 53.5 mm for replicate 1, 57.5 mm for replicate 2, and 63 mm for replicate 3 (Figure 6).

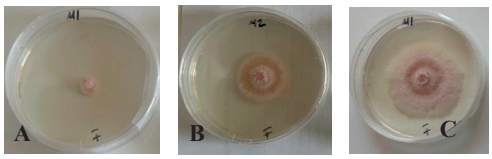

Figure 6. Pathogen evolution at 3 (A), 6 (B) and 9 (C) on control plates (original)

Seventy-two hours after inoculation with *Fusarium* spp., the growth diameters recorded on PDA medium containing discs impregnated with pure essential oil showed limited fungal development. The diameters recorded were 13.5 mm (replicate 1), 15 mm (replicate 2), and 14.5 mm (replicate 3).

At 6 days post-inoculation, the growth continued moderately, with diameters of 27.5 mm, 33.5 mm, and 35 mm for replicates 1, 2, and 3, respectively. After 9 days, further development was observed, with diameters reaching 41 mm (replicate 1), 48.5 mm (replicate 2), and 54.5 mm (replicate 3) (Figure 7).

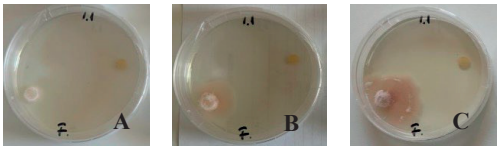

Figure 7. Pathogen evolution at 3 (A), 6 (B) and 9 (C) on discs with pure essential oil

In the experimental variant containing discs impregnated with the commercial volatile oil "S1," fungal growth was observed after 3 days of incubation. The diameters recorded were 10.5 mm (replicate 1), 14.5 mm (replicate 2), and 11.5 mm (replicate 3).

At 6 days post-inoculation, the fungal colony showed further expansion, with diameters of 37.5 mm, 46 mm, and 38 mm for replicates 1, 2, and 3, respectively. By day 9, growth continued, reaching 51 mm (replicate 1), 60 mm (replicate 2), and 57.5 mm (replicate 3) (Figure 8).

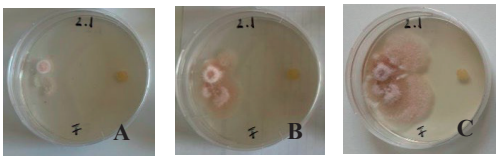

Figure 8. Pathogen evolution at 3 (A), 6 (B) and 9 (C) on discs with commercially available essential oil “S1”

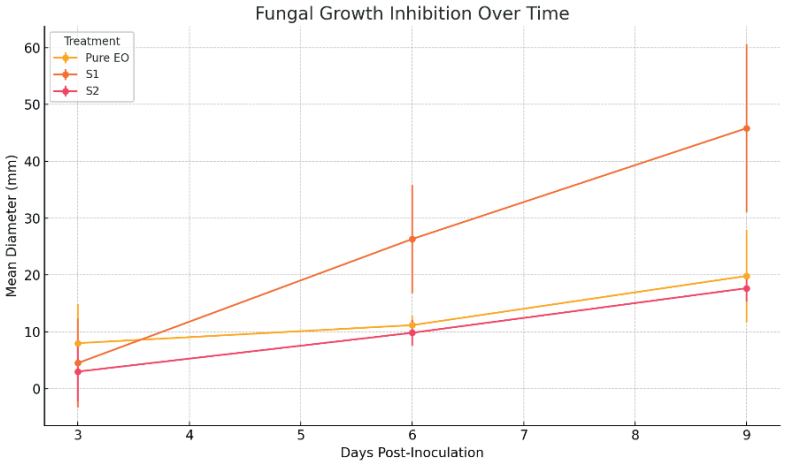

Figure 9. Comparison of *Fusarium* spp. growth on PDA medium treated with different rosemary essential oil samples over 9 days

The graph clearly illustrates that the pure essential oil had the strongest antifungal effect, with significantly lower growth diameters across all time points, while the commercial sample S1 showed a progressive increase in fungal growth, suggesting lower efficacy compared to the pure and S2 samples (Figure 9).

The growth diameter recorded at 72 hours post-inoculation with *Fusarium* spp. in the medium containing discs impregnated with the essential oil extract "S2" ranged from 11 mm (replicate 1) to 13.5 mm (replicate 3), with a mean of 12.3 mm. After 6 days, the mean growth diameter was 30.3 mm, with individual values of 30 mm, 28.5 mm, and 32.5 mm for replicates 1, 2, and 3, respectively. At 9 days post-inoculation, the recorded growth diameters were 43 mm, 36.5 mm, and 47 mm, with a calculated mean of 42.2 mm (Figure 10).

The comparative analysis of the incorporation method using pure rosemary essential oil and two commercial formulations, referred to as "S1" and "S2", revealed significant differences in antifungal activity, likely linked to oil

composition and quality. The term pure essential oil refers to the laboratory-distilled Rosmarinus officinalis oil with no additives or diluents, while "S1" and "S2" are commercial products with unspecified concentrations and possible carriers or solvents.

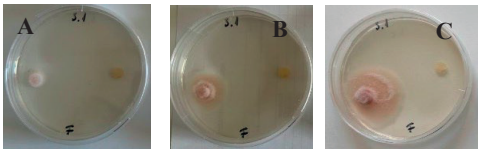

Figure 10. Pathogen evolution at 3 (A), 6 (B) and 9 (C) on discs with commercially available essential oil “S2”

At 3 days post-inoculation, the average growth diameter on PDA supplemented with pure EO was 8 mm, compared to 4.5 mm for S1 and 3 mm for S2. By day 6, the pathogen growth increased to an average of 11.1 mm on pure EO plates, while for S1 it reached 26.3 mm and for S2, 9.8 mm. These results are in agreement with previous studies (Babar et al., 2018; Hussain et al., 2016), which reported higher antifungal effectiveness of undiluted rosemary EO due to its high content of 1,8-cineole, camphor, and α-pinene. Differences between samples may be attributed to variations in oil purity, plant origin, or formulation.Pure essential oil demonstrated its capacity to inhibit pathogen growth throughout the entire 9-day period, with an average diameter of 19.8 mm across the 3 repetitions. In comparison, the "S1" sample recorded an average diameter of 45.8 mm, and the "S2" sample reached an average of 53 mm. The control group showed an average diameter of 63 mm across the 3 repetitions.

These results suggest a notable effectiveness of the 1% solution prepared from pure rosemary essential oil in suppressing the pathogen throughout the observation period. These results suggest that, despite a slower start, pure essential oil exerts a remarkable antifungal effect, making it a viable alternative for managing infections caused by *Fusarium* spp.

The comparative analysis of the diffusion method using filter paper discs between the solution prepared from pure rosemary essential oil (1%) and two commercial oil samples, "S1" and "S2", revealed significant differences in antifungal efficacy. After 3 days, the commercial oils "S1" and "S2" showed slightly higher inhibition, with average growth diameters of 12.16 mm and 12.33 mm, respectively, while the pure essential oil recorded 13.3 mm and the control 13 mm. At day 6, the lowest diameter was observed for "S2" (30.33 mm), followed by the pure essential oil (32 mm), while "S1" and the control presented higher values (36.83 mm and 38.66 mm, respectively). By day 9, although the inhibitory effects decreased overall, "S2" maintained the lowest growth diameter (42.16 mm), followed by the pure oil solution (48 mm). "S1" and the control had the largest diameters (54.16 mm and 58 mm). These results suggest that "S2" and the pure essential oil solution exhibited more sustained inhibitory effects against *Fusarium spp.* compared to "S1" and the control (Figure 11).

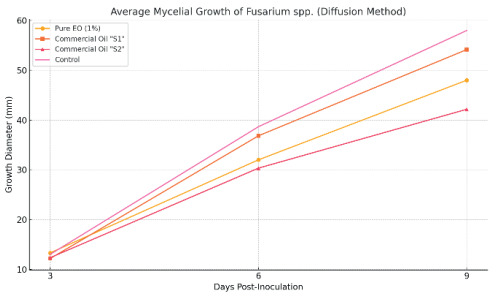

Figure11. Average growth diameter (mm) of Fusarium spp. over 9 days post-inoculation under different treatments using the diffusion method. Commercial oil 'S2' and pure essential oil (1%) demonstrated greater inhibition compared to 'S1' and the control

CONCLUSIONS

In conclusion, the study demonstrated that both the pure rosemary essential oil and the commercial oil "S2" exhibited superior and sustained inhibitory effects on *Fusarium spp.* when compared to the "S1" oil and the untreated control. Although the pure oil showed a slower inhibitory onset, it maintained notable antifungal activity throughout the observation period, indicating potential as an alternative agent in pathogen management.

These results may be attributed to differences in chemical composition among the oils, as the antifungal effectiveness of essential oils is closely linked to their major constituents (e.g., 1,8-cineole, camphor, α-pinene) and

chemotypes. While gas chromatography–mass spectrometry (GC-MS) analysis was not conducted in this study, such characterization is essential in future work to correlate bioactivity with specific chemical profiles. The findings suggest a need for further research to identify the active compounds responsible for the observed antifungal effects and to establish standardized formulations for practical applications.

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