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Research Article

Aspergillus niger antifungal activity of citral and citral - rich Vietnamese balm *Elsholtzia ciliata* essential oil

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Abstract

Mold contamination and mycotoxin production remain major food safety concerns in Vietnam due to climatic vulnerability. With limited access to modern preservation technologies, essential oils (EOs) offer a promising natural strategy for mold control, which aligns with consumer preferences for natural preservatives. This study aimed to evaluate the chemical composition and antifungal activity of Vietnamese balm (*Elsholtzia ciliata*) EO, and to directly compare these properties with those of citral, its major component. Citral, a monoterpene composed of neral and geranial, is recognized as Generally Recognized as Safe (GRAS). Analysis of *E. ciliata* EO from Hong Van, Hanoi, showed high concentrations of citral derivatives (47.12%). Both *E. ciliata* EO and citral inhibited *Aspergillus niger* growth by suppressing mycelial development and delaying sporulation. *E. ciliata* EO displayed a minimum inhibitory concentration (MIC) of 0.34 mg/mL and a minimum fungicidal concentration (MFC) of 5.38 mg/mL, whereas citral's MIC and MFC were lower (0.18 mg/mL and 1.39 mg/mL, respectively), indicating greater potency. Thus, citral alone exhibited stronger antifungal activity than the whole EO, highlighting *E. ciliata* as a valuable natural source for citral extraction and antifungal application.

Keywords: *antifungal activity, citral, Elsholtzia ciliata, essential oil, Aspergillus niger.*

1. INTRODUCTION

Molds are a global and crop-wide problem in food production, posing significant food safety concerns. Among these molds, *Aspergillus niger* is commonly acknowledged as one of the most threatening species. A study reported that *A. niger* was present in 24% of food isolates, 38% of fruit isolates, and 41% of vegetable isolates, making it the most prevalent species identified [1]. The high frequency of *A. niger* was also observed in Vietnam. A survey investigating levels of *Aspergillus* spp. contamination in traditional medicine shops in Hanoi found that *A. niger* exhibited the highest prevalence (61.8%) [2]. *A. niger* was also identified as one of four harmful fungal species present in traditional ramie leaf rice cake samples collected in Binh Dinh Province [3].

Despite the severity of this issue, currently available control methods are not well-suited to the Vietnamese food industry. Physical approaches such as drying, cold storage, or ultraviolet treatment require high capital and operational costs, making them impractical for smallholder farmers, who represent the majority of agricultural producers in Vietnam. Synthetic food preservatives are increasingly rejected by consumers due to concerns about potential health risks and by producers due to fears of inducing antimicrobial resistance. Consequently, essential oils (EOs) extracted from Vietnamese herbs have emerged as promising natural preservatives against fungal contamination, owing to their antimicrobial, antioxidant, and antigenotoxic properties derived from volatile aromatic compounds [4].

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Given that EOs can satisfy both industrial requirements and consumer preferences, increasing attention has been paid to exploring their biological potential. Among these, Vietnamese balm *Elsholtzia ciliata* was selected for investigation due to its long-standing use in traditional culinary and medicinal practices. Previous studies have investigated the chemical profile of *E. ciliata* EO, contributing to the scientific basis of the plant's traditional uses. Analyses of samples collected from different regions of Vietnam, including Long An, Hue, Hanoi, Phu Tho, and Ho Chi Minh City, revealed considerable variability in chemical composition depending on geographic origin and growing conditions. Nevertheless, several major constituents were consistently identified across all samples, including citral (31.3 - 40.2%), β-ocimene (13.3 - 21.5%), and 1-octen-3-ol (4.2 - 7.1%) [5]. These compounds have individually been reported to inhibit the growth of various fungal species through different mechanisms. Among them, Citral, identified as the predominant component of *E. ciliata*, is also a major constituent of EOs derived from *Cymbopogon citratus*, *Litsea citrata*, *Litsea cubeba*, and *Ocimum basilicum* L. Owing to its prominent biological activity, citral has attracted considerable scientific attention, and previous studies have demonstrated that it can effectively reduce microbial contamination and prolong the shelf life of food items [6].

EOs are complex mixtures. Interactions among their constituents may lead to synergistic or antagonistic effects. These effects cannot be fully explained by studying single compounds in isolation or by treating the EO as a homogeneous entity. Therefore, evaluating both the activity of the major compound and that of the entire EO is necessary to better understand their true antimicrobial potential.

Based on these considerations, this study aimed to extract and characterize the chemical constituents of *E. ciliata* EO and to evaluate the antifungal activity of citral and *E. ciliata* EO against *A. niger*, thereby comparing the antifungal efficacy of citral both individually and within the EO matrix, and assessing their potential application as natural antifungal agents.

2. MATERIALS AND METHODS

2.1. Materials

E. ciliata (Vietnamese balm) was collected from Hong Van, Hanoi, Vietnam (20.86352° N, 105.88229° E) at 30 to 40 days old. Citral (purity >95%) was purchased from Sigma Aldrich, USA.

The fungal strain *A. niger* UBOCC-A-112064 used in this study was obtained from the strain collection of Université de Bretagne Occidentale (Brest, France). The strain was activated and propagated in YPD medium (Himedia, India) at 30°C until sporulation occurred.

2.2. Methods

2.2.1. Essential oil extraction by the hydrodistillation method

After harvest, the fresh stems and leaves of *E. ciliata* were thoroughly rinsed with water to remove impurities, then drained. The cleaned plant material was finely chopped into 0.5 - 1 cm segments. All processed plant material was stored at 4°C in the dark and subjected to hydrodistillation within 24 to 48 h to preserve freshness and ensure consistent EO quality. The EO was extracted via hydrodistillation employing a Clevenger-type apparatus. One hundred grams of plant material was mixed with 1200 mL of distilled water (a mass-to-volume ratio of 1/6). The distillation process was performed for 3 h at 100°C. The obtained EO was collected and dehydrated using anhydrous sodium sulfate (Na_2SO_4) before being stored in airtight vials at 4°C [7]. The EO yield was calculated based on the recovered oil volume relative to the mass of fresh basil material and expressed as a percentage (%, v/w).

2.2.2. Gas Chromatography - Mass Spectrometry analysis

The chemical composition of the *E. ciliata* EO was analyzed by gas chromatography-mass spectrometry (GC-MS) using an Agilent 5977B system (Agilent Technologies, USA) at the Lab of Analytical Chemistry, Hanoi University of Science and Technology. The system was fitted with an Agilent 19091S-433UI (HP-5ms Ultra Inert) capillary column (30 m length × 0.25 mm internal diameter; 0.25 µm film thickness). The EO sample was diluted prior to injection. Helium served as the carrier gas, delivered continuously at 1.0 mL/min, while the injection was performed in split mode at 1:25. The oven temperature was programmed as follows: initial temperature held at 60°C for 1 min, then increased to 180°C at a rate of 10°C/min and held for 1 min, finally ramped to 280°C at 16°C/min and held for 5 min. A constant temperature of 250°C was maintained at

the injection port. The mass spectrometer operating parameters were set as follows: ion source temperature, 250°C; ionization energy, 70 eV. The separated components were tentatively identified by comparing their mass spectral data with those in the NIST17.L standard library. The relative percentage composition of each component was calculated based on the peak area integration from the total ion chromatogram [8].

2.2.3. Spot inoculation method

E. ciliata EO and citral were dissolved in 20% Tween 80 (0.5% v/v final concentration) and homogenized to form an emulsion. This emulsion was then incorporated into molten YPD agar (supplemented with 0.5% v/v streptomycin) at 50 to 55°C to achieve final concentration ranges of 0.044 - 0.888 mg/mL (0.005 - 0.1% v/v) for citral and 0.043 - 0.86 mg/mL (0.005 - 0.1% v/v) for the EO. The medium was mixed thoroughly to ensure uniform dispersion. For the spot inoculation assay, fungal spores were collected using a sterile needle and inoculated vertically at the center of each Petri dish containing YPD agar supplemented with the corresponding test concentration. All plates were incubated at 30°C. Control plates were prepared under the same conditions, including a positive control containing amphotericin B (Himedia, India) at 8 mg/L and a negative control (YPD agar without antifungal agents). At each 24-h interval, Colony diameter was determined from two diameters taken in perpendicular directions, after which the average value was used for data analysis. The assay was evaluated until the mycelium in the negative control plates had reached the Petri dish edge. The inhibition rate (IR) was calculated using the formula: inhibition rate (%) = (Dc - De) / Dc × 100%, where Dc is the colony diameter in the control plate (mm), and De is the colony diameter in the treatment plate (mm) [9]. The assay was carried out in triplicate.

2.2.4. Microdilution method

The minimum inhibitory concentration (MIC) is defined as the lowest concentration of an antimicrobial agent that completely inhibits visible microorganism growth [10]. The MIC values of antifungal agents were evaluated through the broth microdilution method on 96-well microplates. Antifungal agents were dissolved and prepared as above. A series of twofold serial dilutions was prepared to produce assay concentration ranges of 0.335 to 43 mg/mL for the *E. ciliata* EO, 0.089 to 11.1 mg/mL for citral, and 0.5 to 64 mg/L for amphotericin B. Each well contained a total volume of 200 µL, consisting of the antifungal solution at the specified concentration, a fungal spore suspension (10^6 spores/mL), and YPD liquid medium. Negative controls (YPD medium only) and positive controls (YPD medium with the fungal spore suspension) were performed under identical conditions. After incubation at 30°C for 48 h, the MIC values were determined. The lower MIC values, the higher antifungal activity, and vice versa. The results were expressed as the mean of three experiments.

The minimum fungicidal concentration (MFC) was determined by transferring 15 µL of the mixture from each MIC well that showed no visible fungal growth onto YPD agar plates before being incubated at 30°C for 24 h. The lowest concentration of the antifungal agent that prevented fungal growth on the agar plates within 24 h was considered the MFC value [10].

2.2.5. Data analysis

The results were expressed as mean ± standard error (SE). Statistical analyses were performed with one-way analysis of variance (ANOVA). A value of $p < 0.05$ was considered statistically significant.

3. RESULTS AND DISCUSSION

3.1. Qualitative analysis of the chemical composition of *E. ciliata* essential oil

The EO of Vietnamese balm (*E. ciliata*) obtained by hydrodistillation of the fresh aerial parts was a light-yellow oil, giving off a characteristic aromatic odor with a pungent flavor. The EO density was 0.895 g/mL, while the yield was 0.3% (v/w).

The chemical compositions of the obtained EO were presented in **Figure 1** and **Table 1**. The results showed that Vietnamese balm (*E. ciliata)* EO contains 34 compounds, of which 32 have been identified, accounting for 99.13% of the total volatile components. The main compounds include: (Z)-Neral (19.80%), (E)-Geranial (27.32%), (E)-β-Farnesene (20.52%), and β-Caryophyllene (8.97%). The main compounds are primarily concentrated in the aldehyde and hydrocarbon groups, among which citral derivatives ((Z)-Neral and (E)-Geranial) are present in high concentrations (47.12%). Citral is also a major component in many EOs such as lemongrass (*C. citratus*), lemon myrtle (*Backhousia citriodora*), and *L. cubeba* EO [11 - 13]. This component

was shown to contribute significantly to both the characteristic aroma and the biological potential of the EO [14]. In addition, citral demonstrates notable antimicrobial potential, particularly toward filamentous fungi and yeasts, through mechanisms that disrupt cell membranes, cause ion imbalance, and inhibit crucial enzymes within fungal cells [15].

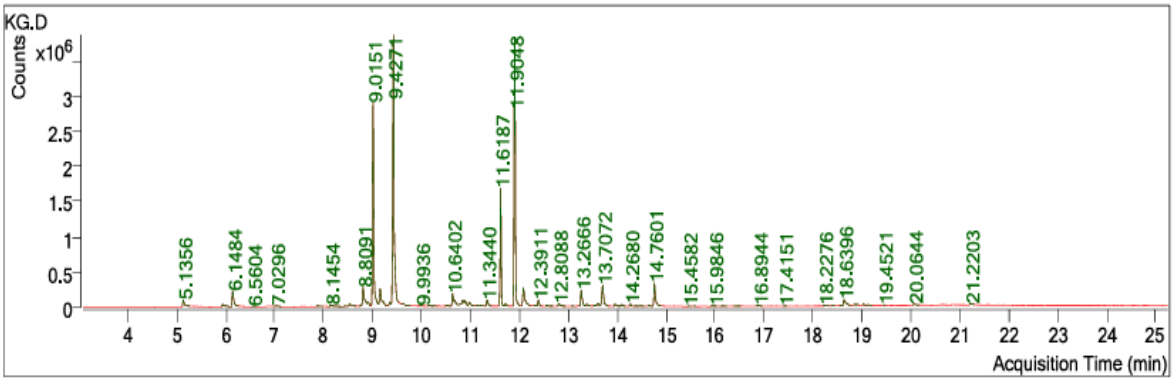

Figure 1. *Chromatogram of E. ciliata essential oil*

Table 1. *Gas chromatography-mass spectrometry result of the E. ciliata essential oil*

No	Retention time	Compounds	% Area	No	Retention time	Compounds	% Area
1	5.14	1-Octen-3-ol	0.83	17	10.85	Bicyclo [4.1.0] heptan-3-ol, 4,7,7-trimethyl-, (1.*α*., 3.*α*., 4.*α*., 6.*α*.)	0.90
2	5.94	Limonene	0.45	18	10.98	1-Undecyn-4-ol	0.49
3	6.15	*β*-Ocimene	1.77	19	11.34	Dodecanal	0.62
4	6.56	Linalyl formate	0.03	20	11.62	*β*-Caryophyllene	8.97
5	7.87	Isoneral	0.14	21	11.72	*(Z)*-*α*-Bergamotene	0.19
6	8.15	Isocitral	0.43	22	11.90	*(E)*-*β*-Farnesene	20.52
7	8.53	Decanal	0.41	23	12.09	Humulene	2.64
8	9.02	*(Z)*-Neral	19.80	24	13.27	*β*-Nerolidol	1.74
9	9.16	*(Z)*-Geraniol	2.47	25	13.71	Caryophyllene oxide	2.66
10	9.36	*(E)*-2-Decenal	0.35	26	13.96	*(Z)*-2-Dodecen-1-ol	0.31
11	9.43	*(E)*-Geranial	27.32	27	14.47	Tau-Cadinol	0.11
12	9.99	*(E)*-Geraniol	0.06	28	14.76	*(Z)*-2-Dodecenal	2.78
13	10.16	*(E)*-Methyl geraniate	0.11	29	15.93	*(E)*-2-Dodecenal	0.07
14	10.52	(-)-*(Z)*-Myrtanyl acetate	0.11	30	18.64	Phytol	0.95
15	10.64	4-Hexen-1-ol, 5-methyl-2-(1-methylethenyl)-, acetate	1.17	31	19.03	Decanoic acid	0.09
16	10.69	n-Decanoic acid	0.54	32	19.13	Geranic acid	0.09

The % area result represents the percentage of a component's peak area on the GC-MS chromatogram relative to the total peak area of all selected/relevant components on the chromatogram

Variations in the chemical constituents of EOs are primarily driven by the plant's genotype, environmental conditions (climate, season, and geography), phenological stage at collection, targeted plant organs, and the choice of extraction technique. Although there is a shared presence of the Citral group including (Z)-Neral and (E)-Geranial - a characteristic potentially specific to the E. ciliata species in Vietnam - the sample analyzed from Thuong Tin exhibited substantially higher concentrations of these compounds compared to results from Thua Thien Hue (with main components being Verbernol (28.87%), Citral (23.65%), and Isocaryophyllene (22.08%)) [16] and Phu Tho ((E)-β-ocimene (19.25%), (Z)-β-farnesene (14.17%), citral (24.13%), limonene (12.58)) [17]. The contrast becomes even more pronounced when compared with data from Northeast China, where the dominant components are entirely different (predominantly Carvone (31.6%), Limonene (22.05%), α-Caryophyllene (15.47%), and Dehydroelsholtzia ketone (14.86%)) [18]. The differences in chemical composition of EOs may affect their biological activity of EOs.

3.2. The antifungal activity of citral and *E. ciliata* essential oil against *A. niger* on solid medium

Mycelial growth inhibition of *A. niger* by *E. ciliata* EO and citral is presented in **Figure 2** and **Figure 3**, respectively. In the control dish, *A. niger* grew normally without contamination, spreading across the whole plate by day 6 and reaching 85 mm in diameter. Sporulation was observed as early as day 2. In contrast, both *E. ciliata* EO and citral significantly reduced colony diameter and delayed spore formation in a concentration-dependent manner. Overall, higher concentrations of either agent resulted in smaller colony diameters and higher inhibition rates.

For citral, the mycelial growth at 0.01% was slower, but the sporulation time remained similar to that observed in the control. At 0.025% and 0.05%, citral delayed sporulation by three days relative to the control. Notably, at the higher concentrations tested, 0.075% and 0.1%, mycelial growth was significantly slower, and sporulation did not begin until the sixth day. Thus, citral effectively inhibited *A. niger* mold by suppressing mycelial development and delaying sporulation on solid medium.

The study by Jian Ju *et al.* (2020) demonstrated that treatment with an eugenol-citral complex significantly altered the morphology and surface structure of *A. niger* hyphae, as observed under scanning electron microscopy (SEM). Untreated hyphae appeared relatively thick with a smooth surface, whereas hyphae treated with the eugenol-citral complex were thinner and exhibited a dry, wrinkled, and even fragmented surface. Notably, the surface morphology of the hyphae was severely altered-they appeared shrunken and collapsed, indicating substantial disruption of both cell wall and membrane integrity [19]. Citral's antifungal mechanism has been elucidated through transcriptomic and proteomic studies. This compound targets the synthesis of ergosterol, a vital constituent of the fungal cell membrane, thereby disrupting cellular integrity. In *Penicillium digitatum*, citral causes membrane damage and disrupts oxidative phosphorylation, while simultaneously altering the expression of numerous proteins (41 over-expressed and 84 repressed). Furthermore, citral has demonstrated a capacity to inhibit growth and sporulation by downregulating the expression of related genes in *A. ochraceus* and *A. flavus* [15].

For *E. ciliata* EO, the same trend was observed. At low concentrations of *E. ciliata* EO (0.01% and 0.025%), the mycelial growth on agar plates was slower than that of the control after 6 days of incubation, but the timing of sporulation showed no significant difference from the control. At higher concentrations of 0.05% and 0.075%, *E. ciliata* EO delayed sporulation by 3 and 5 days, respectively, relative to the control. Notably, at the highest concentration tested, 0.1%, mycelial growth was significantly slower, and sporulation did not begin until the sixth day. Thus, *E. ciliata* EO effectively inhibited *A. niger* mold by suppressing mycelial development and delaying sporulation on solid medium.

When comparing the two treatments, citral generally exhibited a more pronounced inhibitory effect on mycelial growth and sporulation delay than *E. ciliata* EO at comparable concentrations on solid medium, particularly at 0.01% and 0.025% during days 2 and 3 of incubation. To enable a direct and quantitative comparison of antifungal potency, colony diameter was recorded to calculate inhibition rate, and the minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) of both agents were subsequently determined in liquid medium.

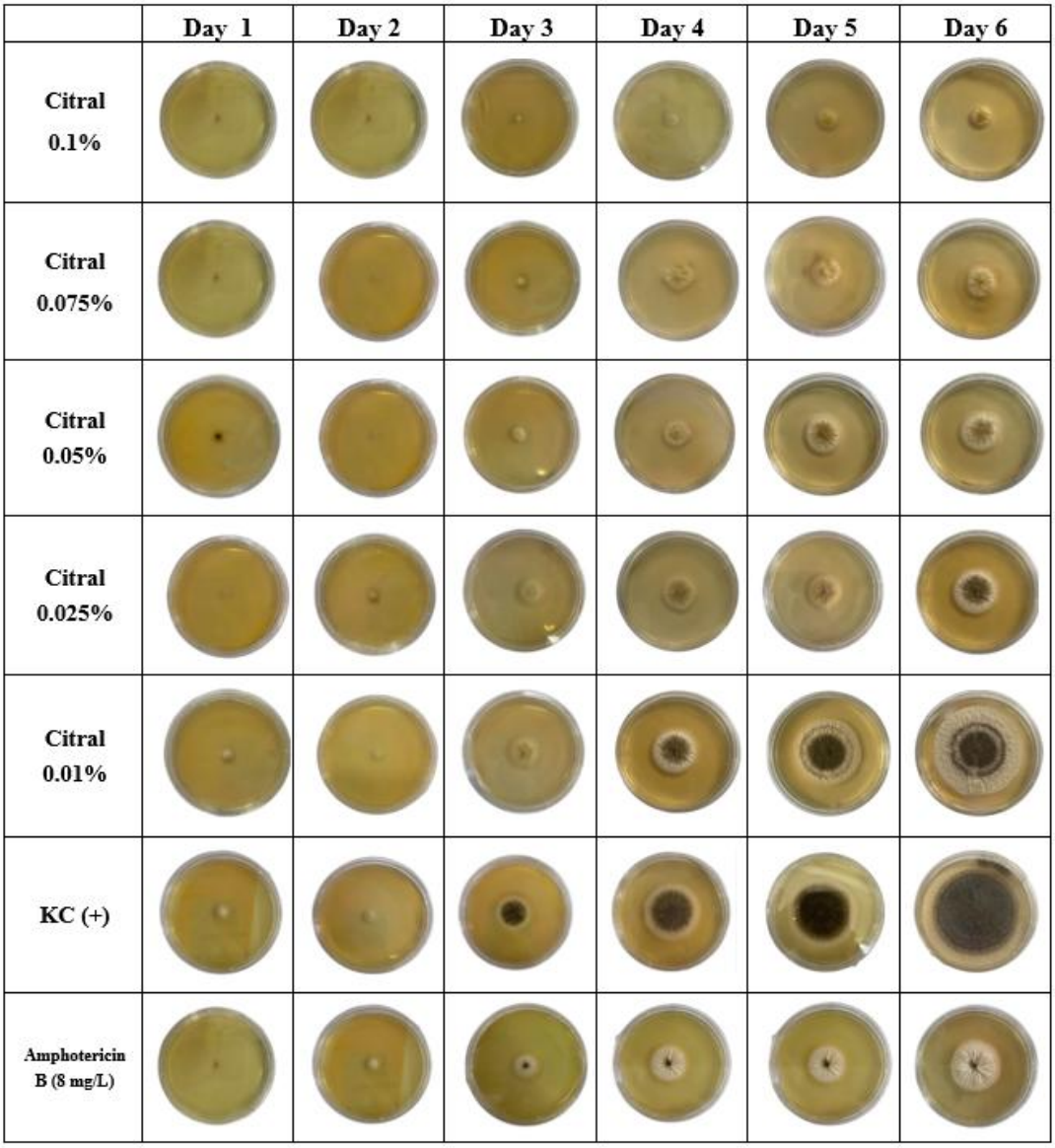

Figure 2. *Antifungal activity of citral and amphotericin B against A. niger on solid medium*

3.3. Impact of natural antifungal on mycelial growth

Growth changes of *A. niger* under *E. ciliata* EO and citral treatments over six days are shown in **Figure 4**. Mycelial growth of *A. niger*, represented by colony diameter, showed a significant decline ($p < 0.05$) as antifungal concentration increased, demonstrating dose-dependent inhibition. The results clearly demonstrate that pure citral exhibited stronger inhibitory effects against mold strain *A. niger* than *E. ciliata* EO at all evaluated concentrations (0.1%, 0.075%, 0.05%, 0.025%, and 0.01%). Notably, even at the lowest concentration of 0.01%, citral maintained substantial inhibition (~ 45%), whereas the effectiveness of the EO declined sharply, approaching a level of minimal activity. Furthermore, comparing with the chemical antifungal agent amphotericin B at a concentration of 8 mg/L, which inhibited the growth of *A. niger* by 62.4%. Both *E. ciliata* and citral demonstrated significant potential as natural alternatives for application in food preservation. However, due to the specific odor of EO and the instability of EO during processing and preservation, some innovative technologies should be used to expand the food applications of these compounds. For example, nanoencapsulation and edible coatings.

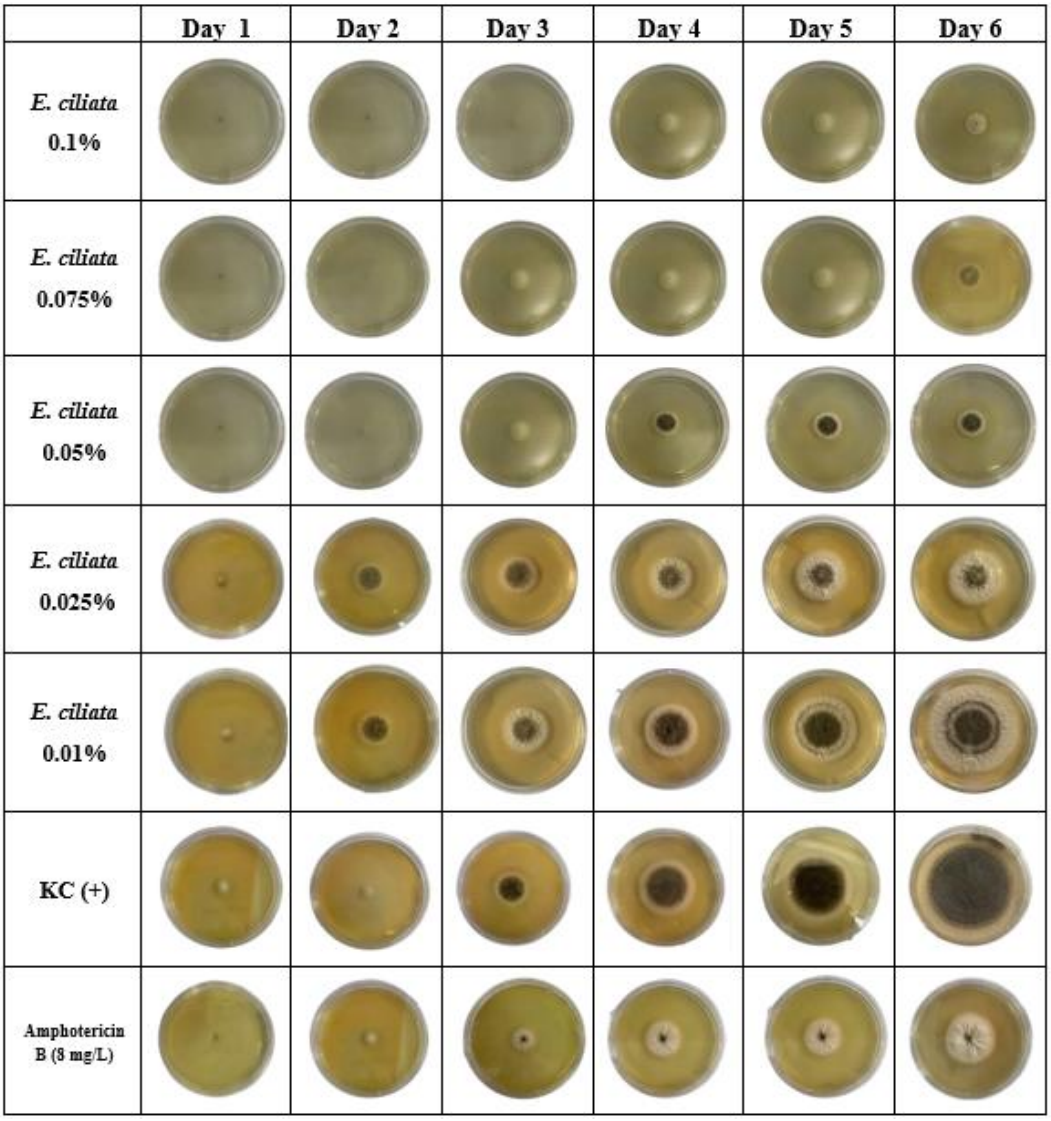

Figure 3. *Antifungal activity of E. ciliata EO and amphotericin B against A. niger on solid medium*

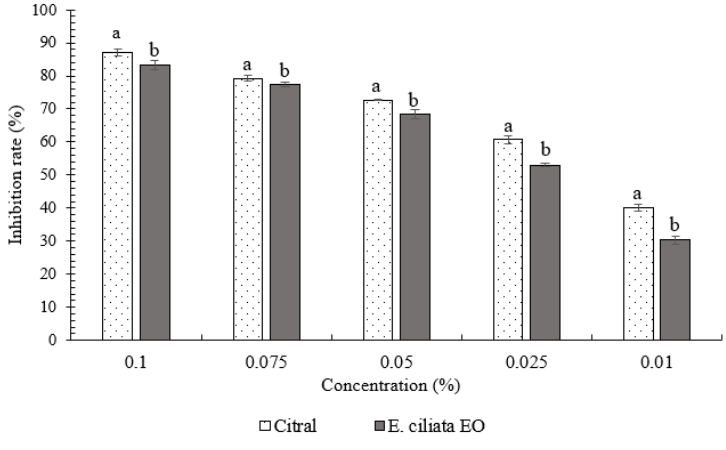

Figure 4. *Impact of citral and E. ciliata EO on the growth of A. niger (Inhibition rate (%) presented as mean ± SE based on triplicate assays)*

3.4. MIC and MFC values

The antifungal capability of *E. ciliata* EO and citral is further supported by the MIC and MFC results obtained in this study. The lower the MIC and MFC values, the stronger the antifungal activity of the antifungal agent, and vice versa (**Table 2**).

Table 2. *MIC and MFC values of E. ciliata EO, citral, and amphotericin B against A. niger*

Values	*E. ciliata* EO	Citral	Amphotericin B
MIC	0.34	0.18	1.6
MFC	5.38	1.39	> 6.4

Note: MIC: Minimum Inhibitory Concentration, MFC: Minimum Fungicidal Concentration, Values for E. ciliata EO and citral are expressed in mg/mL, whereas amphotericin B values are expressed in µg/mL.

The antifungal efficacy of an EO is not solely determined by the presence of a dominant compound like citral, but by the complex interplay of its complete chemical profile. This is evidenced by the comparative activity of citral itself versus citral-rich EOs. In this study, pure citral demonstrated superior activity against *A. niger* (MIC = 0.18 mg/mL, MFC = 1.39 mg/mL) compared to the citral-rich *E. ciliata* EO (MIC = 0.34 mg/mL, MFC = 5.38 mg/mL). This disparity underscores that factors beyond total citral content-such as the synergistic or antagonistic effects of minor constituents, molecular structure, and the balance between lipophilic hydrocarbon frameworks and hydrophilic functional groups-critically influence overall antifungal potency. This principle is further illustrated when comparing different EOs. For instance, *Lippia alba* EO with a very high citral concentration (84.26%) shows strong fungicidal activity (MIC = MFC = 0.6 mg/mL) [20]. In contrast, *L. cubeba* EO, with a broadly comparable citral range (53 to 82%), exhibits a significantly weaker effect (MIC = 1.33 mg/mL, MFC = 5.34 mg/mL). Therefore, while a correlation between citral content and activity may exist, the specific chemical context within each EO ultimately dictates its unique antifungal performance. However, this study tested the antifungal activities of *E. ciliata* EO from Hanoi against *A. niger*, which may limit the generalizability of the findings. Future studies will be essential to a deeper understanding of the antifungal mechanism activity and to expand the applicability of these results.

4. CONCLUSION

In conclusion, this study showed that *E. ciliata* EO contained several key compounds in its composition, including (Z)-Neral (19.80%), (E)-Geranial (27.32%), (E)-β-Farnesene (20.52%), and β-Caryophyllene (8.97%). Among these, citral accounts for the highest proportion (47.12%). Both *E. ciliata* EO and citral effectively inhibit *A. niger* by suppressing mycelial growth and delaying spore formation. Specifically, *E. ciliata* EO and citral inhibited *A. niger* mycelial growth within the concentration range of 0.01 to 0.1%, with inhibition rates of 31.2 - 77.6% and 41.2 - 86.5%, respectively. In liquid medium, MIC and MFC values for *E. ciliata* EO were 0.34 mg/mL and 5.38 mg/mL, respectively, while those for citral were 0.18 mg/mL and 1.39 mg/mL. Overall, citral showed better antifungal activity than citral-rich *E. ciliata* EO. The results suggest that extracting citral from various EOs sources offers a sustainable alternative to synthetic fungicides for food applications.

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