Synergistic Action of the Essential Oils of *Elionorus muticus* and *Lippia junelliana* Against the Causative Agent of Chalkbrood in Bees (*Ascosphaera apis*)

Acción sinérgica de los aceites esenciales de *Elionorus muticus* y *Lippia junelliana* sobre el agente causal de la cría yesificada en abejas (*Ascosphaera apis*)

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ABSTRACT

Ascosphaera apis is the fungus that causes chalkbrood in *Apis mellifera*. Traditionally, its control has relied on synthetic fungicides, which can be harmful to bees and ecosystems. An alternative treatment has explored safer alternatives, such as the antimicrobial properties of plant-based essential oils. This study evaluated the combined effect of the essential oils of *Elionorus muticus* and *Lippia junelliana on* 10 isolates of *A. apis* using a "chessboard" experimental design. Minimum inhibitory concentrations, fungal growth rates and inhibition percentages were determined. Analyses were performed with ANOVA and *post-hoc* tests. The results showed that the combination of 200 µL/L of *E. muticus* and 25 µL/L of *L. junelliana* (C5) completely inhibited fungal growth after 144 h, with MIC_{50} significantly more effective than controls. The combined use of *E. muticus* and *L. junelliana* shows synergistic activity (FICI ≤0.5) in controlling *A. apis* and achieves full inhibition of the fungus under controlled conditions. These findings suggest that essential oils may be a viable alternative for the sustainable management of bee diseases.

Keywords
biocontrol • pathogenic fungi • natural products • apiculture

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Resumen

Ascosphaera apis es el hongo que causa la cría yesificada en *Apis mellifera*. Tradicionalmente, su control ha dependido de fungicidas sintéticos, que pueden ser dañinos para las abejas y el ecosistema. Por ello, se han investigado alternativas más seguras, como los aceites esenciales de plantas, que poseen propiedades antimicrobianas. Este estudio evaluó el efecto combinado de los AEs de *Elionorus muticus* y *Lippia junelliana* sobre diez aislamientos de *A. apis*, utilizando un diseño experimental tipo "tablero de ajedrez". Se determinaron las concentraciones inhibitorias mínimas y se calcularon las tasas de crecimiento fúngico y porcentajes de inhibición mediante ANOVA y pruebas *post hoc*. Los resultados indicaron que la combinación de 200 μL/L de *E. muticus* y 25 μL/L de *L. junelliana* (C5) inhibió completamente el crecimiento del hongo tras 144 horas, con una CIM_{50} significativamente más efectiva que los controles. La combinación de aceites esenciales *E. muticus* y *L. junelliana* presenta una solución prometedora para el control de *A. apis*, con un efecto sinérgico ≥0,5; que permite una inhibición total del hongo en condiciones controladas. Estos hallazgos sugieren que los AEs podrían ser una alternativa viable para el manejo sostenible de enfermedades en abejas.

Palabras clave
biocontrol • hongo patógeno • productos naturales • apicultura

Introduction

Mycotic infections are major contributors to the decline of honey bee (*Apis mellifera* L.) populations, causing significant economic losses in apiculture (6). Among them, the entomopathogenic fungus *Ascosphaera apis* (Maasen ex Claussen) Spiltoir & Olive (1955) causes ascospherosis, or chalkbrood, characterized by dehydrated larvae that change from white to black (depending on the number of sexual reproduction structures present). The infection begins when larvae inadvertently ingest fungal spores with their food (5). Once in larval hindgut, the spores germinate, and subsequent mycelial development causes larval death (22). The fungus affects the bee brood during the pupal stage, reducing the colony's health and productivity (1). Transmission is facilitated by the accumulation of *A. apis* spores in the hive, on beekeeping tools, and in food sources such as nectar and pollen (32).

Currently, ascospherosis is considered a cosmopolitan disease (25). Chemicals are widely used to treat and control chalkbrood, but their use is allowed only in certain countries and under critical conditions. Another alternative is the use of natural products, such as essential oils (EOs) and plant extracts (25). According to some researchers, synthetic fungicides have been linked to increased bee mortality, colony contamination, and reduced beneficial fungi that ferment pollen and support bee nutrition (34). Therefore, natural and ecologically safe products provide a promising option for managing chalkbrood.

Essential Oils (EOs) have long been known to exert antimicrobial effects against pathogens in plants, animals, insects, and honey bees (21, 27). EOs are aromatic substances produced by the secondary metabolism of aromatic plants and can be extracted from leaves, flowers, fruits, seeds, sprouts, rhizomes, roots and bark (23). *Lippia junelliana* (Mold.) Tronc. (Verbenaceae) is an aromatic shrub endemic to the Prepuna and Chaqueña biogeographic regions of Argentina (18). *Elionorus muticus* (Spreng.) is a native grass widely distributed in Argentina and other South American countries (24). Vega *et al*. (2022) determined that 500 μL/L of *L. junelliana* was safe and palatable to honey bees. Albo *et al*. (2014) established the minimum inhibitory concentration (MIC_{50}) of *L. junelliana* EO capable of controlling the chalkbrood pathogen. Up to now, few studies have examined the use of *E. muticus* to control ascospherosis (2).

Mráz *et al.* (2023) suggested that one limitation of EO treatment is the substantial amount of vegetal material required to obtain enough for *in vivo* application. Combining different EOs could address this issue, allowing a reduction in the applied dose in honey bee

colonies. However, most studies have focused on the effects of individual EOs. The present study aimed to evaluate the *in vitro* effects of a combination of *E. muticus* and *L. junelliana* on the pathogenic fungus *A. apis* and to determine the fractional inhibitory concentration index (FICI) of the combinations for potential field application.

Materials and Methods

Isolation and Culture of *A. apis*

Ten *A. apis* isolates were selected for this study due to their reported entomopathogenic capacity in *A. mellifera* brood (table 1) (29). These isolates were stored at -20°C in vials containing 20% glycerol in sterile distilled water and deposited in the Animal Production Course I collection, Facultad de Ciencias Agrarias y Forestales, Universidad Nacional de La Plata (FCAyF, UNLP). For fungal inoculum, isolates of *A. apis* were cultured on Petri dishes with MY20 medium (5 g peptone, 3 g yeast extract, 3 g malt extract, 200 g glucose and 20 g agar per liter of distilled water) at 35°C for 7 days, following the methodology of Albo *et al*. (2016).

Table 1. Fungal isolates of *A. apis* and their geographical origin.

Tabla 1. Aislamientos fúngicos de *A. apis* y su origen geográfico.

Isolated #	County	Province	Country
44	General Paz	Buenos Aires	Argentina
99	La Plata	Buenos Aires	Argentina
73	Florencio Varela	Buenos Aires	Argentina
76 (Aa31)*	Punta Indio	Buenos Aires	Argentina
77 (Aa40)	Tandil	Buenos Aires	Argentina
39 (Aa41)	Unión (Justiniano Posse)	Córdoba	Argentina
75 (Aa39)	Marcos Juárez (Monte Buey)	Córdoba	Argentina
98 (Aa35)	Guatraché	La Pampa	Argentina
26	Neuquén	Neuquén	Argentina
8 (Aa8)		Valdivia (Region X)	Chile

*Isolation number assigned by Reynaldi *et al.*, 2003.

*Número de aislamiento asignado en Reynaldi *et al.*, 2003.

Essential Oils

The EOs of *E. muticus* and *L. junelliana* were provided by the Aromatics Group, Institute of Instituto Nacional de Tecnología Agropecuaria (INTA), Castelar, Buenos Aires, Argentina. The EOs were extracted from plant tissue via Clevenger hydrodistillation (IRAM Standard No. 18.729) and stored in brown glass vials at -20°C for later use. Vega *et al.* (2022) described the chemical characterization of the EOs. The main components of *L. junelliana* are cis- and trans-ocimenone, myrcenone, cis-davone, and camphor, while those of *E. muticus* are geranial, neral, geranyl acetate, linalool, and methyl heptenone.

Experimental Procedure

To assess the effect of the combination of EOs on the entomopathogenic fungus, the “chessboard” method was used (13). Initial concentrations of 200 µL/L for *E. muticus* and 400 µL/L for *L. junelliana* were selected based on preliminary trials (unpublished data). The experimental design, shown in figure 1 (page 4), included five decreasing concentrations in a geometric series. Dilutions of the EOs were prepared using 2.5% v/v ethanol at 70°C as a diluent, which was then added to the melted MY20 liquid medium (45°C) and poured into Petri dishes to create each combination. Approximately 20 ml of medium was poured

into glass Petri dishes (9 cm x 1.5 cm). Mycelium discs (7 mm diameter) from each fungal isolate were placed on the medium surface with the different EO/diluent combinations in duplicate. As a control, we inoculated each strain in MY20 medium. All plates were incubated at 35°C, 12% CO_2, 65% relative humidity, and in darkness for 144 h. The fungal growth diameter (mm) was measured with a caliper every 24 h.

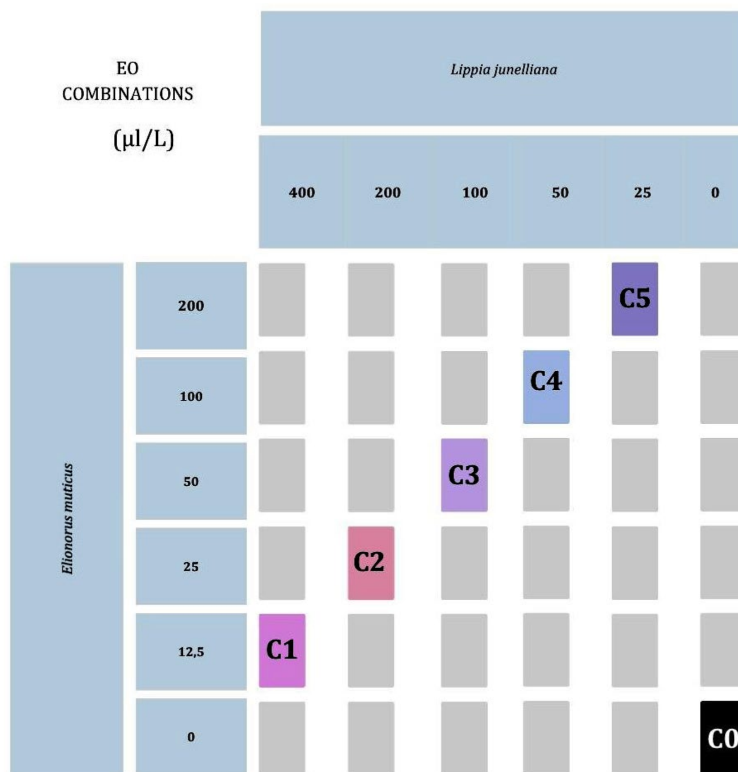


Figure 1. Chessboard. The matrix was structured with rows representing *E. muticus* concentrations and columns representing *L. junelliana* concentrations. Each box at the row-column intersection represents a combination of *E. muticus* and *L. junelliana.*

Figura 1. Tablero de ajedrez. La matriz se estructuró utilizando filas (Concentraciones de *E. muticus*) y columnas (Concentraciones de *L. junelliana*), en la cual cada casilla (intersección de fila y columna) representa una combinación de *E. muticus* y *L. junelliana.*

Growth Parameters

Based on the data obtained, the absolute growth was calculated for each culture time, and growth curves were constructed. The exponential growth rate per hour (GR; mm/h) was determined using the following equation 1:

$$\mathbf{TC = \sum (DC_C - DC_T)/DC_C \times 100\%} \tag{1}$$

DC_C corresponds to the control growth diameter and DC_T to the treatment growth diameter.

$$\mathbf{TC = C24h + C48h + C72h + C144h/4} \tag{2}$$

$$\mathbf{C24h = total\ mycelium\ length - 7} \tag{3}$$

$$\textbf{C48h = (total mycelium length - 7) - C24h} \quad (4)$$

$$\textbf{C72h = (total mycelium length - 7) - C48h} \quad (5)$$

$$\textbf{C144 h = (total mycelium length - 7) - C72h} \quad (6)$$

ANOVA followed by Tukey's post-hoc test ($p = 0.05$) was used to determine differences among the GRs for each treatment.

Descriptive statistics were calculated for the final fungal growth diameter at the end of the experiment (144 h). ANOVA followed by Holm-Sidak's test was used to determine differences between the control and EOs combinations. Then, Tukey's post-hoc test ($p \leq = 0.05$) was used to determine differences among EO combinations, excluding the control.

Percent Growth Inhibition

The percentage of growth inhibition (GI) was calculated according to Chen *et al*. (2019) using the following formula (Eq. 7):

$$IC (\%) = (DC_C - DC_T)/DC_C \times 100\% \quad (7)$$

Where DC_C is the control growth diameter and DC_T is the treatment growth diameter.

A bifactorial ANOVA followed by Tukey's *post hoc* test ($p \leq 0.05$) was conducted to assess differences among EO combinations and culture times on growth inhibition percentage. Before statistical analysis, percentage data were transformed using arcsine $\sqrt{p}$.

Minimum Inhibitory Concentration (MIC_{50})

To assess the efficacy of the combination of EOs against the entomopathogenic fungus, the number of inhibited strains relative to the control was calculated (10). The MIC_{50} was determined at the time points of the linear growth phase (72 and 144 h). It was defined as the lowest EO concentration causing 50% or more inhibition compared to the control (4).

Fractional Inhibitory Concentration Index (FICI)

The FICI value was calculated from the results obtained for MIC_{50} using the following equations (36):

$$\textbf{FICi = FICa + FICb} \quad (8)$$

$$\textbf{FICa = A/MICa} \quad (9)$$

$$\textbf{FICb = B/MICb} \quad (10)$$

A, MIC of EO A in combination; MIC_a, MIC of EO A alone; B, MIC of EO B in combination; MIC_b, MIC of EO B alone.

FICI values were interpreted according to Amassmoud *et al.* (2023) as follows: synergism ($FICI \leq 0.5$), additive ($0.5 < FICI \leq 1.0$), neutral ($1 < FICI \leq 4.0$), or antagonism ($FICI > 4.0$).

Results

Figure 2 (page 6), shows the growth curves and rates of *A. apis* for the EO combinations and control. All EO combinations inhibited fungal growth during the logarithmic phase. *A. apis* required more than 72 h to overcome the fungistatic effect produced by C4 (100 μL/L *E. muticus* and 50 μL/L *L. junelliana*). Fungal growth was not observed when exposed to C5 (200 μL/L *E. muticus* and 25 μL/L *L. junelliana*) after 144 h of culture. All EO combinations showed growth rates below 50% compared to the control ($F: 102.29; p < 0.001$).

Different letters in the growth rate values indicate significant differences among treatments (Tukey's test, $p < 0.05$).

Letras distintas en los valores de tasa de crecimiento indican diferencias significativas entre los tratamientos (Test de Tukey, $p < 0,05$).

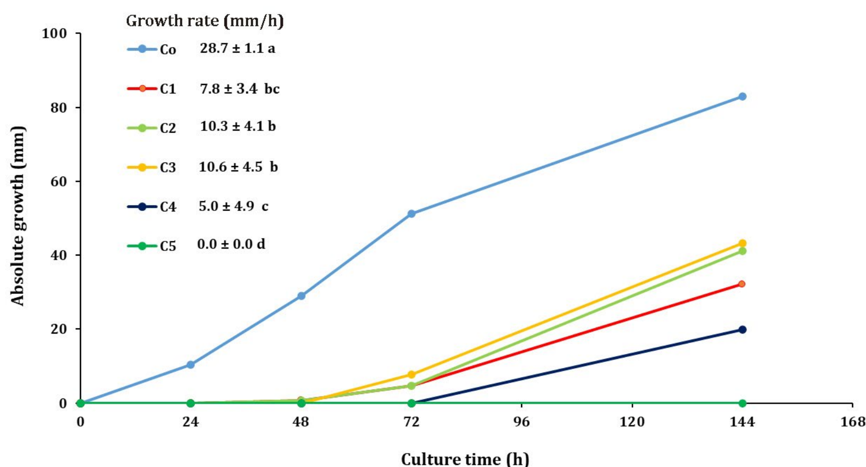


Figure 2. Growth curves and rates of *A. apis*.

Figura 2. Curvas y tasas de crecimiento de *A. apis*.

Table 2, shows descriptive and parametric analyses of *A. apis* growth at the final time point (144 h) after exposure to the EO combinations. ANOVA revealed significant differences among treatments (F: 136.08; $p < 0.0001$), with each EO combination differing from the control with a highly significant p value ($p \leq 0.001$, Holm-Sidak's test). Growth was strongly affected by combination C5, followed by C4 (Tukey's test, $p < 0.05$).

Table 2. Descriptive statistics and *post-hoc* parametric analysis of EO combinations on *A. apis* growth diameter (mm) at 144 h of culture.

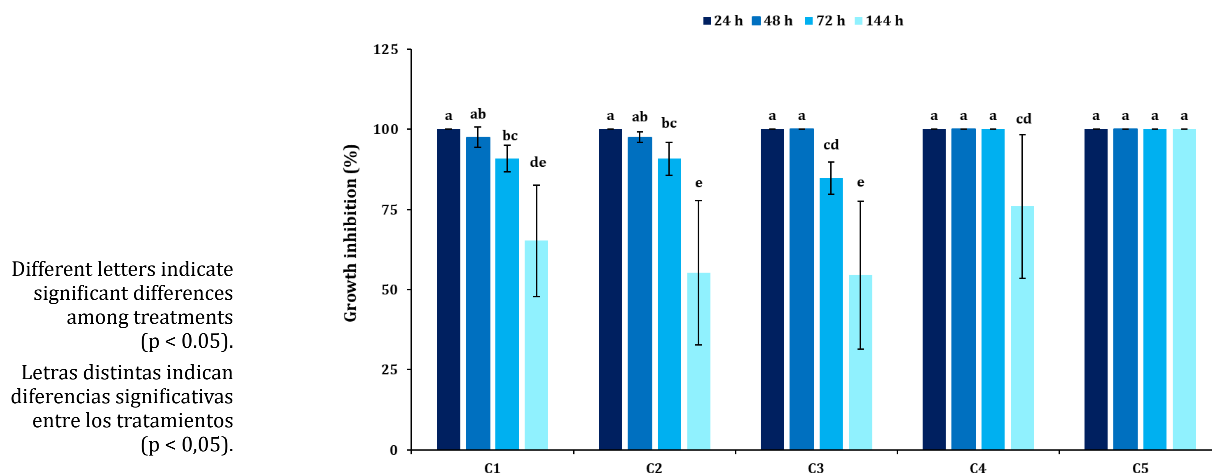
Tabla 2. Estadística descriptiva y análisis paramétrico *post-hoc* de la combinación de los AEs sobre el diámetro de crecimiento (mm) de *A. apis* a las 144 h de cultivo.

Treatment	Co	C1	C2	C3	C4	C5
Minimum	26.0	0.0	0.0	0.0	0.0	0.0
Maximum	30.0	13.0	18.5	18.3	14.0	0.0
Average	28.0	7.8	10.3	10.6	6.3	0.0
Deviation	1.1	3.9	4.1	4.9	4.5	0.0
Holm-Sidak's test [a]		<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
Tukey's test [b]		ab	a	a	b	c

a p value obtained. b Different letters indicate significant differences among treatments ($p < 0.05$).

a, valor de p obtenido. b, Letras distintas indican diferencias significativas entre los tratamientos ($p < 0,05$).

The relative growth inhibition of *A. apis* showed significant differences (F: 9.01; $p < 0.001$), depending on the EO combination and culture time (figure 3, page 7). All EO combinations and culture times inhibited 50% of fungal growth; however, C5 was the only one that achieved 100% inhibition at all time points.



Different letters indicate significant differences among treatments ($p < 0.05$).

Letras distintas indican diferencias significativas entre los tratamientos ($p < 0,05$).

Figure 3. Growth inhibition (%) of *A. apis* the EO combinations.

Figura 3. Inhibición porcentual del crecimiento de *A. apis* en las diferentes combinaciones de los Aceites esenciales.

The relative growth inhibition of *A. apis* strains at each culture time is shown in table 3. The percentage of affected strains (< 50%) decreased as culture time increased for combinations C1, C2, C3, and C4, while C5 maintained inhibition of 50% or more at all time points. Based on the MIC_{50} values, the FICI was calculated. The FICI for C5 was 0.5, indicating that the combination of both EOs exerted a synergistic effect that led to the total inhibition of *A. apis* strains.

$$FICA = 200/400 = 0.5$$
$$FICB = 25/3200 = 0.007$$
$$FICi = 0.5 + 0.0 = 0.5$$

Table 3. Relative percentage inhibition of *A. apis* strains at different culture times.

Tabla 3. Porcentaje relativo de inhibición del número de cepas de *A. apis* en los distintos tiempos de cultivo.

Time (H)	C1	C2	C3	C4	C5
24	100	100	100	100	100
48	40	20	100	100	100
72	0	0	0	100	100
144	0	0	0	40	100

Discussion

Essential oils have well-known antimicrobial properties and are used to treat infections caused by several pathogens (5). Previous studies (briefly described below) have examined *Lippia junelliana* and *Elionurus muticus* separately against pests and pathogens, but never in combination. This study investigated the inhibitory effects of combining *L. junelliana* and *E. muticus* against *A. apis*.

The EO of *L. junelliana* is mainly composed of myrcenone and cis-davanone (33). Comelli *et al*. (2023) reported strong insecticidal activity against *Oryzaephilus mercator* (L.) (Nitidulidae), with LC_{50} values of 7.02–7.17 µL/L and LC_{100} values of 10.00-20.00 µL/L. El Youssfi *et al*. (2024) demonstrated that this EO exhibits virucidal activity against the hemorrhagic yellow fever virus (Junín virus), with a MIC_{50} (VC_{50}) of 20 ppm. Regarding its antifungal activity, *in vitro* tests showed that *L. junelliana* effectively controlled molds and yeast, including *Candida krusei, C. albicans*, and *Aspergillus spp.* of the section *fumigati* (13). Regarding pests and pathogens in beekeeping, Dellacasa *et al*. (2003) found that *L. junelliana* repelled the mite *Varroa destructor* at 1.9 µL per bee. The inhibitory effect of this EO against *A. apis* was studied alone but never in combination (15).

The *E. muticus* essential oil used in this study was mainly composed of approximately 50% α-citral (geranial) and 25% β-citral (neral) (33). *In vivo* tests demonstrated its fumigant activity against the agricultural pest *Tetranychus urticae* C.L. Koch (Acari) at low concentrations (0.03-1 µL/L) (17). Regarding its bactericidal activity, *E. muticus was* effective against *Escherichia coli, coagulase-negative Staphylococcus, S. aureus, S. pseudintermedius, S. uberis, P. aeruginosa,* and *Bacillus cereus*, with MIC values ranging from 1 to 150 mg/mL (8). Fungicidal activity was observed against *Candida albicans* (9), *C. krusei*, and *C. neoformans* Vuillemin (20), with MIC values of 0.5-5 µg/mL. Fungistatic activity was reported against *C. parapsilosis* and *C. utilis* (28).

Davis and Ward (2003) determined that the minimum fungicidal concentration against *A. apis* was 250 ppm for *C. citratus*, *Eucalyptus citriodora*, and *Leptospermum petersonii* oils, all rich in citral. Similarly, the essential oil of *Litsea cubeba* (Lour.) Pers (Lauraceae) showed fungicidal activity against *A. Apis* at 50 µg/mL using the agar diffusion method (5, 26). Dellacasa *et al.* (2003) reported that 800 ppm of *Tagetes minuta*, which shares components with *L. junelliana*, was required to control *Ascosphaera apis in vitro*. In our study, the combination of *E. muticus* (200 µL/L) and *L. junelliana* (25 µL/L), referred to as C5, produced a FICI of 0.5, showing the most effective control of *A. apis* through synergistic action. This effect likely resulted from the complementary interaction of the oils' active components, yielding greater antifungal efficacy than either oil used alone. This finding aligns with recent research showing that synergistic essential oil combinations can fully inhibit various fungal pathogens (35). According to previous studies, citral, a major component of the *E. muticus* EO used here, effectively inhibited both dematiaceous fungi and the hyaline fungus causing chalkbrood (7, 29). Our combination of *L. junelliana* and *E. muticus* reduced the required *L. junelliana* amount by at least 75% compared to the 800 ppm reported by Dellacasa *et al.* (2003) for *Tagetes minuta*, which contains similar major components.

It is important to highlight the time-dependent effects of the essential oil combinations on *A. apis*. Combination C4 (100 µL/L of *E. muticus* and 50 µL/L of *L. junelliana*) showed a fungistatic effect, requiring more than 72 h for the fungus to recover. However, it did not fully inhibit fungal growth. Analysis of relative inhibition percentages and their temporal variation revealed significant differences in the efficacy of the essential oil combinations. Combinations C1, C2, C3, and C4 showed reduced effects over time, whereas C5 consistently maintained inhibition above 50% throughout the study. Sustained complete inhibition is desirable for controlling fungal pathogens, providing more effective and long-lasting protection (20). The synergistic effect and temporal stability observed make C5 a promising candidate for controlling *Ascosphaera apis*.

Conclusions

Our results showed that combining *L. junelliana* (25 µL/L) and *E. muticus* (200 µL/L) synergistically inhibited *A. apis* growth. This combination outperformed the inhibitory efficacy of each oil alone and reduced the required amount of *L. junelliana* by at least 75% compared to previously reported values, lowering the costs of potential health management. Although our results provide valuable information on *in vitro* control dosages, further studies on the toxicity of the tested compounds in larval, pupal, and adult honey bees are needed to successfully apply this knowledge in field conditions.

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