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Dirección de Postgrado
Facultad de Agronomía
Programa de Magíster en Ciencias Agronómicas

NANOEMULSIÓN DE ACEITE ESENCIAL DE *Laureliopsis philippiana* (Losser) Schodde: ACTIVIDAD INSECTICIDA CONTRA LA POLILLA DEL TOMATE (*Tuta absoluta* Meyrick)

Tesis para optar al grado de Magíster en Ciencias Agronómicas

ANA MARÍA SALAZAR PROBOSTE
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Profesor Guía: Nelson Zapata San Martín
Dpto. de Producción Vegetal
Facultad de Agronomía
Universidad de Concepción

Esta tesis ha sido realizada en el Departamento de Producción Vegetal de la Facultad de Agronomía, Universidad de Concepción.

Profesor Guía

Dr. Nelson Zapata San Martín
Facultad de Agronomía
Universidad de Concepción

Comisión Evaluadora:

Dra. María Dolores López Belchi
Facultad de Agronomía
Universidad de Concepción

Dr. Nolberto Arismendi Solis
Centro de Investigación Austral Biotech
Universidad Santo Tomás

Director de Programa

Dra. Marisol Vargas Concha
Facultad de Agronomía
Universidad de Concepción

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TABLA DE CONTENIDOS

ÍNDICE DE FIGURAS Y TABLAS

fumigation. Significant differences between treatments were estimated by a Factorial ANOVA (Factor 1: Type of formulation (EO and NE); Factor 2: Concentrations (0, 10, 20, 40, 60 $\mu L\ L^{-1}$). Different letters in the bars indicate significant differences between treatments (Tukey HSD test, $p<0.05$).

RESUMEN

Tuta absoluta Meyrick constituye una grave amenaza para la producción de tomate en el mundo. Las actuales estrategias de control incluyen el uso de aceites esenciales (AEs) y sus formulaciones. *Laureliopsis philippiana* (Losser) Schodde, especie arbórea nativa de Chile, cuyo AE tiene reconocida actividad biológica frente a otras plagas, podría tener efecto insecticida sobre *T. absoluta*. Por ello los objetivos de este estudio fueron determinar la estabilidad de una nanoemulsion (NE) a base de AE de *L. philippiana* y evaluar el efecto insecticida de la NE y el AE puro contra *T. absoluta*. La estabilidad de la NE se midió durante un período de 35 días, con mediciones realizadas cada 7 días, mientras que la eficacia de la NE para controlar *T. absoluta* se comparó con la del AE puro. Los resultados indicaron que la NE se mantuvo estable durante todo el período de evaluación, promediando al final del experimento un tamaño de partícula de 40,60 nm, un potencial zeta de -14,54 mV, un índice de polidispersión (PDI) de 0,30 y un nivel de pH de 4,64. La NE de *L. philippiana* tras 4 horas de fumigación mostró una mayor actividad insecticida contra *T. absoluta* que el AE puro, con valores de CL_{50} y CL_{90} de 4,6 y 27,8 µL L^{-1}, mientras que el AE puro dio lugar a valores de 27,1 y 75,3 µL L^{-1} respectivamente. Estos resultados corresponden al primer reporte de las propiedades insecticidas del AE de *L. philippiana* contra *T. absoluta*. La NE resultó mucho más eficiente que el AE puro sobre todo a bajas concentraciones y se mantuvo estable por más de 4 semanas.

SUMMARY

Tuta absoluta Meyrick is one of the most serious pests of tomatoes worldwide. The current strategies of pest control for *T. absoluta* include essential oils (EOs) and its formulations. Biological activity of *Laureliopsis philippiana* (Losser) Schodde EO, a tree species native from Chile, has been demonstrated against pests. Hence, the aims of this study were to determine the stability of the *L. philippiana* oil- based nanoemulsion (NE) and to evaluate the insecticidal effects of pure and NE forms of the EO against *T. absoluta*. Emulsion stability was measured over a 35-day period, with measurements conducted every 7 days, while the efficacy of the NE to control *T. absoluta* was compared to that of the pure EO. The results indicate that the NE remained stable during the whole evaluation period, averaging at the end of the experiment a particle size of 40.60 nm, zeta potential of – 14.54 mV, polydispersity index (PDI) of 0.30 and a pH level of 4.64. The *L. philippiana* NE after 4 h fumigation showed greater insecticidal activity against *T. absoluta* than pure EO, with LC_{50} and LC_{90} values of 4.6 and 27.8 µL L^{-1}, whereas the EO resulted in values of 27.1 and 75.3 µL L^{-1}, respectively. These results are the first report of the insecticidal properties of the EO of *L. philippiana* against *T. absoluta*. The biological activity exerted by the oil was significantly enhanced when developed as a NE as it remained stable for more than four weeks and was much more efficient in the control of *T. absoluta* than pure EO, particularly at low concentrations.

CAPÍTULO 1

INTRODUCCIÓN GENERAL

El tomate (*Solanum lycopersicum* L.) es una de las frutas procesadas y frescas más importantes del mundo y el segundo cultivo hortícola más importante después del poroto a nivel mundial (Costa y Heuvelink, 2018). Dentro de las plagas de importancia económica que afectan al tomate en el mundo, se encuentra la polilla del tomate, *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae), la cual afecta a cultivos bajo plástico y al aire libre (Campolo et al., 2017), causando pérdidas de un 50 a 100% de rendimiento (Gebremariamd, 2015). En 10 años, esta plaga pasó de infestar un 3% a un 60% de la superficie cultivada de tomate a nivel mundial, convirtiéndose en la mayor amenaza para países como China y Estados Unidos; países que se encuentran entre los tres mayores productores de tomate a nivel mundial (Biondi et al., 2018).

Debido al hábitat endófito de *T. absoluta*, es decir, que las larvas se alimentan y se distribuyen en el interior del mesófilo de las hojas, tallos y frutos; les permite estar protegidos de la mayoría de los compuestos químicos (Biondi et al., 2018). Este comportamiento conlleva a fomentar el uso de insecticidas sintéticos, lo que resulta en la pérdida de eficacia (Silva et al., 2011) y la resistencia a éstos con diferentes modos de acción (Roditakis et al., 2018). Además, el uso excesivo de insecticidas genera impactos negativos en los agentes de biocontrol, facilitando el resurgimiento de plagas secundarias, y dejando residuos en el medio ambiente (Silva et al., 2011), pero también en los frutos que son consumidos y/o comercializados en un rango de tiempo relativamente corto en donde no permite que los productos aplicados se degraden por completo (Jallow et al., 2017). Por lo tanto, es necesario encontrar nuevas alternativas para controlar *T. absoluta*, tales como, por ejemplo, el desarrollo e implementación de insecticidas botánicos (Essoung et al., 2017). Estos afectan a la plaga con pocos o ningún efecto secundario, no solo matando al insecto plaga, sino que afectando su fisiología de varias maneras como la sobrevivencia, el comportamiento, el desarrollo, reproducción y vías metabólicas (Piri et al., 2020). Dentro de estos compuestos químicos naturales útiles para el control de plagas, los aceites esenciales (AEs) de las plantas aromáticas son uno de los grupos más importantes (Regnault- Roger et al., 2012). Los AEs se producen fácilmente por destilación al vapor del material vegetal y contienen muchos fenoles volátiles de bajo peso molecular, monoterpenos, sesquiterpenos y fenilpropanoides que son tóxicos para muchas plagas de insectos (Regnault- Roger et al., 2012; Isman y Grieneisen, 2014; Piri et al., 2020).

Existen varios estudios que han evaluado la actividad insecticida de AEs de plantas sobre *T. absoluta* reportando buenos resultados en su control (Umpierrez et al., 2017; Adil et al., 2015; Campolo et al., 2017; Chegine y Abbasipour, 2017; Chegine et al., 2018; Rahmani y Azimi, 2021; Liambila et al., 2021). En este sentido, la flora nativa de Chile posee una enorme diversidad de especies, constituyendo una rica fuente de AEs, con especies con probada actividad insecticida contra diversas plagas de insectos (Bittner et al., 2008; Betancur et al., 2010; Zapata y Smagghe, 2010; Torres et al., 2014; Norambuena et al., 2016; Pinto et al., 2016, Zapata et al., 2016; Arias et al., 2017). Dentro de estas especies nativas el efecto insecticida de *Laureliopsis philippiana* (Looser) Schodde ha sido notable, reportándose efectos tóxicos por contacto, fumigantes, repelentes y anti- alimentarios, principalmente sobre plagas de granos almacenados (Herrera et al., 2015; Norambuena et al., 2016; Bustos et al., 2017; Ortiz et al., 2017). Sin embargo, no existen estudios previos que informen sobre la actividad biológica de *L. philippiana* contra la polilla del tomate. Los análisis fitoquímicos de tres especies arbóreas nativas de Chile pertenecientes a la familia Monimiaceae, incluyendo *L. philippiana*, mostraron que 1,8-cineol, safrol y metileugenol son compuestos comunes en estas especies y que poseen antecedentes documentados de actividad insecticida en otros AEs de plantas (Herrera et al., 2015; Ortiz et al., 2017). Algunos de estos compuestos se han utilizado para controlar *T. absoluta* (Chegine y Abbasipour, 2017), lo que sugiere que el AE de *L. philippiana* podría tener efectos insecticidas contra esta especie.

Sin embargo, los insecticidas botánicos presentan ciertos inconvenientes asociados como alta volatilidad, baja solubilidad en agua y tendencia a la oxidación (Moretti et al., 2002; Choupanian et al., 2017). Las nanoformulaciones pueden resolver estos problemas, ya que pueden mejorar la estabilidad de los

AEs, evitando la degradación y las pérdidas por evaporación, permitiendo una liberación controlada y facilitando su manipulación (Werdin et al., 2014). Las nanoformulaciones que han sido desarrolladas en el último tiempo se han clasificado como nanosuspensiones, nanoemulsiones y nanoencapsulaciones, cada una desarrollada de acuerdo a la planta y a la plaga que se quiera controlar (Kumar et al., 2019). De hecho, las nanoemulsiones (NE) pueden mejorar las propiedades y la eficacia de los bioinsecticidas de uso comercial (Anjali et al., 2012), permitiendo una alta solubilidad en agua y la capacidad de solubilizar compuestos hidrofílicos y lipofílicos, lo que conlleva el uso de menos ingredientes inertes, químicos y activos, lo que a su vez ayuda a reducir los costos de producción (Choupanian at al., 2017). En este sentido y pensando en que *T. absoluta* daña principalmente la parte aérea de la planta, desarrollar una NE presenta ventajas, ya que se puede acceder directamente al área de la planta donde se encuentra la plaga y permitir un contacto directo al utilizar el agua como medio de dispersión. Es por esto que los objetivos de este estudio fueron determinar la estabilidad de una nanoemulsión basada en el aceite de *L. philippiana* y evaluar el efecto insecticida tanto de la NE como del AE puro sobre *T. absoluta*.

HIPÓTESIS

El aceite esencial de *L. philippiana* tiene efecto insecticida sobre *T. absoluta* y al ser formulado como nanoemulsion éste incrementa su eficacia sobre la plaga, además de mantenerse estable por más de cuatro semanas.

OBJETIVO GENERAL

Evaluar el efecto insecticida del aceite esencial de *L. philippiana* y su nanoemulsión sobre *T. absoluta*, y determinar la estabilidad de la nanoemulsión mediante la evaluación de parámetros fisico- químicos.

OBJETIVOS ESPECÍFICOS

1. Diseñar una nanoemulsión en base a aceite esencial de *L. philippiana.*

2. Determinar la estabilidad de la nanoemulsión de *L. philippiana* mediante la evaluación de parámetros físico- químicos

3. Evaluar el efecto insecticida del aceite esencial de *L. philippiana* y su nanoemulsión sobre larvas L2 de *T. absoluta*.

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CAPITULO 2

Nanoemulsion of *Laureliopsis philippiana* (Looser) Schodde essential oil: Insecticidal activity against tomato borer (*Tuta absoluta* Meyrick)

Ana María Salazar [a], Nolberto Arismendi [b], María Dolores López [a], Marisol Vargas [a], Mauricio Schoebitz [a], Daniel A. Palacio [c], José Becerra [d], Braulio Cedeño [a], Nelson Zapata [a]*

[a] Faculty of Agronomy, University of Concepción, Av. Vicente Méndez 595, Chillán, Chile.

[b] Austral Biotech Research Center, Santo Tomás University, Av. Ramón Picarte 1130-01160, Valdivia, Chile.

[c] Polymers Department, Faculty of Chemical Sciences, University of Concepción, Casilla 160-C, Concepción, Chile.

[d] Botany Department, Faculty of Natural Sciences and Oceanography, University of Concepción, casilla 160- C, Concepción, Chile.

***Corresponding author**: Nelson Zapata San Martín, Faculty of Agronomy, University of Concepción, Av. Vicente Méndez 595, Chillán, Chile. E-mail: nzapata@udec.cl

Keywords: Essential oil, Fumigant toxicity, Nanoemulsion stability, Bioinsecticide

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Abstract

Tuta absoluta is one of the most serious pests of tomatoes worldwide. The current strategies of pest control for *Tuta absoluta* include essential oils (EOs) and its formulations. Biological activity of *Laureliopsis philippiana* essential oil, a tree species native from Chile, has been demonstrated against pests. Hence, the aims of this study were to determine the stability of the *L. philippiana* oil-based nanoemulsion (NE) and to evaluate the insecticidal effects of pure and NE forms of the essential oil against *T. absoluta*. Emulsion stability was measured over a 35-day period, with measurements conducted every 7 days, while the efficacy of the NE to control *T. absoluta* was compared to that of the pure EO. The results indicate that the NE remained stable during the whole evaluation period, averaging at the end of the experiment a particle size of 40.60 nm, zeta potential of – 14.54 mV, polydispersity index (PDI) of 0.30 and a pH level of 4.64. The *L. philippiana* NE after 4 h fumigation showed greater insecticidal activity against *T. absoluta* than pure EO of, with LC_{50} and LC_{90} values of 4.6 and 27.8 µL L^{-1}, whereas the EO resulted in values of 27.1 and 75.3 µL L^{-1}, respectively. These results are the first report of the insecticidal properties of the EO of *L. philippiana* against *T. absoluta*. The biological activity exerted by the oil was significantly enhanced when developed as a NE as it remained stable for more than four weeks and was much more efficient in the control of *T. absoluta* than pure EO, particularly at low concentrations. The use

of *L. philippiana* essential oil for sustainable agriculture presents a promising strategy in the seek of new insecticide products.

Keywords: Essential oil, Fumigant toxicity, Nanoemulsion stability, Bioinsecticide

1. Introduction

Tomato (*Solanum lycopersicum* L.) is one of the most important fresh and processed fruits and the second most important vegetable crop after bean at a global scale (Costa and Heuvelink, 2018). Tomato borer, *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae) is a serious insect pest of tomato, which affects crops under greenhouse and open field conditions (Campolo et al., 2017), causing high yield reductions that range from 50 to 100% (Gebremariamd, 2015) and resulting also, in great economic losses. In 10 years, the infestation of this pest increased from affecting 3% to 60% of the cultivated area worldwide, becoming the greatest threat to countries like China and the United States, which are among the three largest producers of tomato worldwide (Biondi et al., 2018).

Due to the endophytic habitat of T. absoluta, which is found inside the mesophyll of the leaves, stems and fruits; protects this insect from most chemical compounds (Biondi et al., 2018), encouraging the use of synthetic insecticides, resulting in efficacy loss (Silva et al., 2011) and resistance to insecticides with different modes of action (Roditakis et al., 2018). Additionally, the use of insecticides is generating negative impacts on biocontrol agents, facilitating the resurgence of secondary pests, and leaving residues in the environment (Silva et al., 2011) and in the fruit, which is commercialized and consumed within a short time. (Jallow et al., 2017). Therefore, there is a need to find new alternatives to synthetic chemical insecticides such as botanical insecticides in order to allow for an effective and safe control of *T. absoluta* (Essoung et al., 2018). As these are naturally occurring chemicals, they pose little or no threat to the environment or humans, killing the insects they are meant to control, and having effects on their physiology in terms of survival, behavior, development, and reproduction (Piri et al., 2020). Within these natural chemical compounds that are useful for pest control, the essential oils (EOs) from aromatic plants are the one of the most important groups (Regnault- Roger et al., 2012). EOs are easily produced by steam distillation of plant material and contain many volatile, low-molecular-weight phenolics, monoterpenes, sesquiterpenes, and phenylpropanoids that are toxic for many insect pests (Regnault- Roger et al., 2012; Isman and Grieneisen, 2014; Piri et al., 2020).

There are several studies that have reported on the insecticidal activity exerted by EOs extracted from plants against *T. absoluta* (Umpierrez et al. 2017, Adil et al. 2015, Campolo et al. 2017, Chegine and Abbasipour, 2017; Chegine et al., 2018; Rahmani and Azimi, 2021; Liambila et al., 2021). In this sense, the native flora of Chile has an enormous diversity of species, constituting a rich source of

EOs, with species with proven insecticidal activity against various insect pests (Bittner et al., 2008; Betancur et al., 2010; Zapata and Smagghe, 2010; Torres et al., 2014; Norambuena et al., 2016; Pinto et al., 2016, Zapata et al., 2016; Arias et al., 2017). Several studies have described the insecticidal effects of *Laureliopsis philippiana* (Looser) Schodde, reporting contact, fumigant, repellent and antifeedant activity against insects, particularly against stored grain pests (Herrera et al., 2015; Norambuena et al., 2016; Bustos et al., 2017; Ortiz et al., 2017). However, there are no previous studies reporting on the biological activity of *L. philippiana* against tomato borer. Phytochemical analyses of three tree species belonging to the Monimiaceae family (*L. philippiana* included), native to Chile, have showed that they contain compounds such as 1,8-cineol, safrole and methyleugenol, which are commonly found in essential oils (EOs) from these species (Herrera et al., 2015; Ortiz et al., 2017). Some of these compounds have been used to control *T. absoluta* (Chegine and Abbasipour, 2017), suggesting that the EO of *L. philippiana* might have insecticidal effects against this species.

Despite their promising properties, however, botanical insecticides exhibit high volatility, low water solubility and a tendency to oxidation (Moretti et al., 2002; Choupanian et al., 2017). Nanoformualtions can solve these problems as they can enhance the stability of EOs, preventing degradation and evaporation losses, allowing a controlled release, and facilitating their handling (Werdin et al., 2014). In recent years, nanoformulations developed to control different types of pests have been classified as nanosuspensions, nanoemulsions and nanoencapsulations (Kumar et al., 2019). In fact, nanoemulsions (NE) can enhance the properties and efficacy of bioinsecticides for commercial use (Anjali et al., 2012), allowing for a high solubility in water and the ability to solubilize hydrophilic and lipophilic compounds, which leads to the use of less inert, chemical and active ingredients, which in turn helps reduce production costs (Choupanian at al., 2017). Considering that *T. absoluta* mainly damages the aerial part of the plant, the use of a NE would allow for a direct access to the area of the plant where the pest is found and a more direct contact by using water as a dispersion medium. Thus, the aims of this study were to determine the stability of the *L. philippiana* oil- based NE and to evaluate the insecticidal effects of pure and NE forms of the EO against *T. absoluta*. Emulsion stability was measured over a 35-day period, with measurements conducted every 7 days, while the efficacy of the nanoemulsion to control *T. absoluta* was compared to that of the pure EO.

2. Materials and Methods

2.1. Extraction of EO from L. philippiana

The EO was extracted from the leaves samples of *L. philippiana* collected during the spring of 2020 in Los Maitenes, Río Bueno locality, Los Ríos Region (40°25’44.3” S, 72°29’12.7” W), Chile. The leaves were randomly selected from each cardinal points of the central part of the trees. The collected material was identified by comparison with the reference voucher (CONC-CH237) from the herbarium of the University of Concepción, School of Agronomy, University of Concepción, Chillán, Chile. Once collected, the material was taken to the Phytochemical Laboratory, School of Agronomy, University of Concepción, Chillan, Chile. The leaves were washed and dehydrated to constant weight in a drying chamber (Binder, FD-23, Germany) at 40°C ± 1 °C. Once dried, the leaves were crushed to an approximate size of 0.5 to 1 cm^{-2} using a grinder (Moulinex, AD6011CL, Colombia). The EO was extracted from the crushed material by steam distillation for two hours, using a Clevenger-type equipment, obtaining an oil yield of 3% P/V. The extracted oil was dried with anhydrous sodium sulfate (Na_2SO_4) and then stored at 4°C ± 1 until use.

2.2. Preparation of the L. philippiana EO-based nanoemulsion

The nanoemulsion of *L. philippiana* EO was prepared according to Mazarei and Rafati (2019) with some modifications, using the high-speed homogenization method. For this, 3 % of *L. philippiana* EO and 9 % of surfactants were used (60% corresponded to Tween® 80 (Sigma Aldrich, USA) and 40 % corresponded to Span® 80 (Sigma Aldrich, USA)). Two phases were prepared, an aqueous phase consisting of distilled water (88%) and Tween 80 and an oily phase consisting of *L. philippiana* EO and Span 80. The aqueous phase solution was emulsified using a high-speed homogenizer (IKA T18 digital Ultra-Turrax®, Dispersing tool S18 N-10G, Brazil) at 15.000 rpm for two minutes, and then poured into the oily phase. Subsequently, the oil-in-water emulsion was again emulsified by high-speed homogenization at 15,000 rpm for another 5 minutes. The nanoemulsion was maintained at a room temperature of 20 ± 4 °C.

2.3. *Chemical characterization of pure EO and EO-based nanoemulsion of L. philippiana*

The chemical composition of the EOs was determined using a gas chromatograph (GC, Agilent 7890, USA) coupled with an HP-5MS capillary column (length of 30 m, inner diameter of 0.25 mm, thin-film of 0.25 µm and injection volume of 1 µL). The initial temperature of the equipment was 60 °C, which was maintained for 5 minutes, with an increase of 10 °C every minute until 280 °C, where it was held for 15 minutes. The relative percentages of the compounds in the pure EO and nanoemulsion *of L. philippiana* were obtained using helium as the mobile phase and a mass selective detector (Agilent 5975C, USA). The components were identified using the NIST/EPA/NIH Mass Spectral Library (NIST 17).

2.4. *Physical-chemical characterization of pure EO and EO-based nanoemulsion of L. philippiana*

Particle size, polydispersity index (PDI), and zeta potential of the nanoemulsion were determined by photon correlation spectroscopy and laser Doppler anemometry using a Zetasizer NanoBrook 90Plus particle size analyzer (Brookhaven Instruments Corp., USA), with a 35 mW high-power diode laser, scattering angle of 90°, laser wavelength of 659 nm and temperature of 25 °C. All analyzes were performed using a cell with a 1-cm path length, while temperature was calibrated at each measurement. pH measurements and control were carried out with a Mettler Toledo pH meter (SevenCompact, pH/Ion S220, China), which was previously calibrated with a buffer solution at different pH levels (2.0, 5.0, 7.0, 9.0, and 11.0), while coefficients of correlation ($R2=$ 0.998) were obtained.

The nanoemulsion was maintained at room temperature (20 ± 4°C). Measurements were made every 7 days for 35 days. On each day of evaluation, a sample was taken from the nanoemulsion and at least 3 readings were taken for each parameter evaluated.

2.5. *Insecticidal activity of pure EO and EO-based nanoemulsion of L. philippiana against T. absoluta.*

2.5.1 Insect rearing and plant material

During the entire study period, tomato plants were produced from seeds cv. San Pedro in order to guarantee the presence of insects. The plants were kept in a greenhouse at an average temperature of 28 ± 2 °C.

The population of *T. absoluta* used in the experiment was obtained from an infested commercial tomato orchard located in the Huape locality, Chillán, Chile. Rearing was maintained for two years in tomato plants in a bioclimatic chamber with a photoperiod of 16/ 8 h (light/dark), at an average temperature of 28 ± 2 °C and a relative humidity (RH) of 60 ± 5 %. Depending on the environmental conditions during rearing, L2 instar larvae between 2 and 2.5 mm in size were used (Salazar and Araya, 2001).

2.5.2. Fumigation chambers

The *L. philippiana* EO and EO-based nanoemulsions were applied in fumigation chambers, which consisted of 500-mL glass flasks with a sealing lid. A pad consisting of a 2 × 2 cm Whatman® grade-2 absorbent paper square was placed inside the lid, to which the desired concentration of pure EO and nanoemulsion was applied. Then the flasks were immediately covered and sealed with Parafilm M® (Heathrow Scientific, USA).

A 1.5 mL tube was placed inside each flask and fixed to the bottom with plasticine, and the petiole of a tomato leaf was added. Then the larvae of *T. absoluta* were placed inside to feed on the leaves. The experiment was conducted under light/dark conditions (16/8 h), at a temperature of 28 ± 2 °C and a relative humidity of 60 ± 5 % (RH).

2.5.3. *Dose-response bioassay*

The EO of *L. philippiana* was applied at concentrations of 5, 10 ,20 ,40 and 60 µL L^{-1} of air, whereas the EO-based nanoemulsion was applied at concentrations of 2.5, 5, 10, 15, 20, 40 and 60 µL L^{-1} of air. Control treatments (untreated) were also included.

The dose-response bioassay experiments were conducted in 4 replicates per treatment, using ten *T. absoluta* L2 instar larvae for each replicate.

Then, evaluations were made 4 and 8 h after fumigation and the number of dead larvae in each unit was counted. Larvae were considered dead when they showed no movement when gently touched with a camel-hair brush. LC_{50} and LC_{90} were calculated according to Finney (1971).

2.6 *Data analysis*

A repeated measures analysis of variance (Repeated Measured ANOVA) was used for all the parameters evaluated in the physical-chemical characterization of the nanoemulsion, with at least 3 replicates for each evaluated variable. Subsequently, a Tukey HSD test was performed for comparison of means ($p<0.05$). Also, a factorial analysis of variance (Factorial ANOVA) was performed to evaluate the mortality of *T. absolute* larvae by fumigation under different concentrations and type of formulation (pure EO and nanoemulsion), while separation of means was carried out using the Tukey HSD test ($p<0.05$).

All the analysis was performed using the statistical software Statistica 7.0 (StatSoft, USA).

Additionally, a probit analysis was performed to estimate LC_{50} and LC_{90} values with 95% confidence intervals (CI) by POLO Plus V 2.0. CL values are significantly different when their 95% CIs do not overlap, but also, chi- square and p- values were also reported in this analysis.

3. Results and discussion

3.1. Chemical characterization of pure EO and EO-based nanoemulsion of L. philippiana

EO-based nanoemulsion and pure EO were identified by GC- MS. For the pure EO, 97.29 % of its compounds were identified (Table 1). The compounds with the highest concentrations were linalool (38.11 %), safrole (28.20 %), 1,8-cineole (12.77 %) and methyleugenol (5.75 %), all belonging to the chemical family of monoterpenes and phenylpropanoids. These results coincide with a study *on L. philippiana* EO conducted by Niemeyer and Teillier, 2007, who reported similar concentrations of the same compounds: linalool (43.4 %), safrole (21.4 %), 1,8- cineole (8.2 %) and methyleugenol (10 %). Furthermore, Herrera et al. (2015) and Bustos et al. (2017) also coincide with the chemical composition but reported different content levels, being safrole the compound with the highest concentration (39.56%), followed by linalool (34.45 %), 1,8-cineole (8.28 %), and methyleugenol (3.06 % and 4.47, respectively). These variations could be attributed to the fact that, even though the plant material used comes the south of Chile, the collection sites were different. The leaf samples used by Niemeyer and Teillier (2007), Herrera et al. (2015) and Bustos et al. (2017) were collected in the Los Lagos Region, while those used in the present study were collected in the Andean sectors of Río Bueno, Los Ríos Region. In this sense, studies conducted by Herrera et al. (2015) and Ortiz et al. (2017) concluded that the chemical composition of *L. philippiana* is affected by the geographical area where it grows and by the time of year where the plant material is collected. For instance, Bittner et al. (2009) collected leaves in the Bío Bío Region in autumn, while we collected leaves in the Los Ríos Region in spring. Bittner et al. (2009) also reported that the most abundant compound was 3-carene (53.81 %,) followed by 1,8-cineole (14.76 %) and 1,2-dimethoxy-4-(2-propenyl)-phenol (10.58 %), and thus 1,8-cineole was the only compound found in similar concentrations in both studies.

The chemical composition of *L. philippiana* EO contained in the nanoemulsion was determined in up to 96.57 %, detecting no presence of sesquiterpenes, caryophillene, cis-calamenene and germacrene. All the other compounds present in the pure EO were found in the nanoemulsion with the same values of retention time (min) and area (%) (Table 1). Therefore, apart from containing the EO in its matrix, the nanoemulsion maintained the chemical composition of the pure EO.

However, some of these compounds may present some toxicity such as safrole. Therefore, emphasis should be placed in future studies on safety issues for mammalian health, non-target organisms and the environment in order to promote the practical application of the EO and EO- based nanoemulsion of *L. philippiana* (Regnault- Roger et al., 2012).

3.2. *Physical-chemical characterization of the EO-based nanoemulsion of L. philippiana*

One of the main problems in the development of nanoemulsions are stability problems, therefore in this study the NE developed were investigate and characterized to be stable over the time. The formulation was characterized over a 35-day period in terms of particle size, PDI, zeta potential and pH.

Statistically significant differences were found in the size of nanoemulsion particles as a function of the measurement time ($F = 35.92$; $p < 0.001$; df = 5, 20). The nanoemulsion particles varied in size during the 35 days of the experiment. Initially the particles were smaller, reaching a size of 29.35 nm on day 1. Later, these particles increased in proportion, reaching their largest size (49.84 nm) on day 21 (Figure 1). After this period, the nanoemulsion particles tend to reduce in size, although with sizes greater than those seen at the beginning of the experiment (Figure 1), which could indicate that the system tends to remain stable. In addition, the average particle size did not exceed 50 nm, which is well below the value of 200 nm considered by some authors as the maximum droplet size for an emulsion to be referred as a nanoemulsion (Mazarei and Rafati, 2019; Gharsan et al., 2022).

The size obtained may be associated with the oil: surfactant ratio used (1: 3). Choupanian et al. (2017) found that an amount of surfactant greater than 1.5 times that of EO is required to obtain particles of less than 100 nm, which is what happened in the present study. Regarding stability, Anjali et al. (2012) found that nanoemulsions were stable when oil and surfactant were used at a 1:3 ratio. This result was similar to reported by Kale and Allen (1989), who found that the addition of surfactant to nanoemulsions causes the interfacial film to condense and stabilize, resulting in a small droplet size and a more stable system. Therefore, based on the sizes obtained over the 35-day period and the oil: surfactant ratio used, it can be stated that the EO-based emulsion corresponds to a nanoemulsion, which tended to keep stable over the evaluation period since particle size did not exceed the value of 49.84 nm recorded on day 21 (Figure 1).

The PDI of the nanoemulsion showed significant differences between measurements during the evaluation period. ($F = 4.13$; $p = 0.010$; df = 5, 20). The PDI values showed an increasing trend from day 1 to 35, with values ranging from 0.23 to 0.30. However, no significant differences were found between days 1 and 28, nor between the measurements made between days 14 and 35, indicating that the nanoemulsion system presents particle size homogeneity and stability. In fact, PDI values below 0.20 indicate a narrow size distribution, and thus a good long-term stability to the nanoemulsion (Sugumar et al., 2014; Gharsan at al., 2022). In the present study, the nanoemulsion remained stable over the evaluation period because the PDI averaged values close to 0.20 (Figure 2).

Significant differences were recorded in the evaluation period ($F = 5.04$; $p = 0.014$; df = 5, 10) in the Zeta potential, with average values between -29.13 mV and -7.4 mV, recorded on days 28 and 14, respectively. Significant differences were recorded in the evaluation period ($F = 5.04$; $p = 0.014$; df = 5, 10) (Figure 3). However, no differences were observed in this parameter between day 1, 7 and day 35. This indicates that, despite the variations observed between the measurements, Zeta potential values remained similar on the first days and at the end of the experiment, which shows the stability of the system. In addition, the means of Zeta potential values in this study were far from zero, which would indicate that the nanoemulsion remains stable, since values close to zero indicate flocculation (Pinto and Buss, 2020). This can be explained by the Brownian motion because high values of zeta potential result in a low sedimentation rate and prevents a rapid phase separation (Adak et al., 2020). In the present study, this helped maintain emulsion stability, which correlated with nanometric sizes and low PDI values.

There were significant differences the measurements over the evaluation period according to pH level ($F = 75.70$; $p < 0.001$; $df = 5, 10$). The pH values ranged between 4.62 on day 1 and 4.76 on day 21. However, no differences were found in the pH level between day 1, 28 and 35, indicating that pH remained stable at the beginning and the end of the study period (Figure 4). This occurs because pH is an important determinant of emulsion stability since changes in pH level suggest ongoing chemical processes that could affect the quality of the system and the compounds (Ishaka et al., 2014). In terms of pH, the results can be correlated to the oil: surfactant ratio used (1:3). This agrees with the results reported by Adak at al. (2020) for a nanofumulation obtained using the same ratio. The authors reported a pH value of 4.86 and found that lower ratios result in lower pH values, suggesting that the decrease or increase in pH values is related to the increase or decrease in the content of surfactants (Moretti et al., 2002; Choupanian et al., 2017; Adak et al 2020).

3.3. *Insecticidal activity of the pure EO and EO-based nanoemulsion of L. philippiana against T. absoluta*

The efficacy of the EO-based nanoemulsion was compared to that of the pure EO of *L. philippiana* by fumigation. There were significant differences between treatments ($F = 12.91$; $p = 0.001$; $df = 4, 30$), which responds to an interaction between the type of formulation (pure EO and EO- based NE) and the concentration (0, 10, 20, 40 and 60 µL L^{-1} of these compounds present in the fumigation chambers. Mortality rate of *T. absoluta* L2 instar larvae recorded 4 h after fumigation was much higher with the EO- based nanoemulsion compared with the pure EO at all the low concentrations (10, 20 and 40 µL L^{-1}) (Figure 5). Nevertheless, similar mortality of insects was obtained at the highest concentration of 60 µL L^{-1} with both the pure EO and the EO- based nanoemulsion. This indicates that the *L. philippiana* nanoemulsion is much more efficient against *T. absoluta* than the pure EO because a lower concentration of EO would be required to obtain higher mortality, which is clearly evidenced in the results obtained for LC_{50} and LC_{90} (Table 2). In fact, there were no detected significant differences between LC_{50} and LC_{90} with respect to pure EO and EO-based nanoemulsion. However, LC_{50} and LC_{90} values of EO- based NE were lower than EO pure (Table 2). These results indicate that efficacy of EO of *L. philippiana* increases when it is formulated as a NE, in some cases reducing the use of pure EO more than four times. This agrees with the results obtained by

Adak et al. (2020) and Gharsan et al. (2022), who reported a more than two-fold increase in efficacy when pure EO was formulated as a nanoemulsion.

The insecticidal effect observed in both the pure EO and EO- based nanoemulsion can be attributed to the high content of monoterpenoids found in *L. philippiana* oil. In fact, it has been described that monoterpenoids are the main compounds with insecticidal effects in EOs extracted from plants (Regnault-Roger and Hamraoui, 1995; Ahn et al. 1998). They are volatile and lipophilic substances that can penetrate the cuticle of the insect rapidly and interfere with its physiological functions. Due to their high volatility, monoterpenoids have fumigant and gaseous actions (Ahn et al., 1998; Lee et al., 2003). This has been confirmed in some studies carried out in Chile that have reported insecticidal and repellent properties in the EO of *L. philippiana* against stored grain insects (Herrera et al., 2015; Norambuena et al., 2016; Bustos et al., 2017; Ortiz et al., 2017). In addition, some of the compounds identified as the most abundant in *L. philippiana* EO have also been identified in EOs extracted from other plant species, which have exhibited insecticidal and repellent properties against *T. absoluta*, as is the case of 1,8-cineol, linalool and methyleugenol (Chegine and Abbasipour, 2017; Essoung at al., 2018).

The mortality of *T. absoluta* produced by the nanoemulsion showed that the chemical compounds present in the EO of *L. philippiana* play a fundamental role in its insecticidal effect, being comparatively higher than that of pure EO (Figure 5), which could be explained by the fact that nanoparticles improve both stability and effectiveness of botanical insecticides. In fact, nanoformulations can protect active compounds from degradation and evaporation losses, improving their efficacy (Werdin et al. 2014; López et al., 2021), and thus volatile compounds could have been active much longer during fumigation. On the other hand, the small particle size of nanoemulsions increases the penetration and absorption of EO active compounds in the insect body, which can improve the biological activity of nanoemulsions compared to pure EOs (Adak et al., 2020).

4. Conclusions

The results of this study show that the essential oil of *L. philippiana* has a high insecticidal effect on *T. absoluta*. However, the EO of *L. philippiana* formulated as a nanoemulsion can be much more effective against this pest, achieving higher mortality rates at much lower concentrations than the pure EO.

According to the parameters evaluated, the EO-based nanoemulsion of *L. philippiana* was stable at room temperature over the 35-day evaluation period. The physical-chemical analysis showed that the pure EO and the EO contained in the nanoemulsion have the same chemical composition, with high concentrations of linalool, safrole, 1,8-cineole and methyleugenol.

The efficacy of EO-based nanoemulsion of *L. philippiana* against *T. absoluta* was demonstrated. However, further standardization is required in order that it can be a competitive alternative to synthetic insecticides. In this sense, future research needs to evaluate its insecticidal activity against other stages of pest development, including bioassays on non- target organisms and field applications.

Authors’ contributions

NA, MDL, MV, DP and MS contributed to conceptualization and methodology. AMS and BC performed all experiments. NA, NZ, AMS, JB and DP analyzed the data. AMS wrote the original draft preparation. All authors contributed critically to the drafts and gave final approval for publication.

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Table 1

Chemical composition of essential oil and nanoemulsion of *L. philippiana*

Compound	Retention Time (min)	Area (%)	
		EO	NE
α- pinene	5.729	1.10	1.10
sabinene	6.497	0.74	0.74
β- pinene	6.559	1.47	1.47
β- myrcene	6.829	0.80	0.80
α- phellandrene	7.099	2.30	2.30
o- cymene	7.493	1.58	1.58
1,8- cineole	7.643	12.77	12.77
β- ocimene	7.944	0.46	0.46
γ- terpinene	8.157	0.25	0.25
terpinolene	8.738	0.71	0.71
linalool	9.060	38.11	38.11
4- terpineol	10.456	0.22	0.22
α-terpineol	10.689	1.49	1.49
safrole	12.484	28.20	28.20
eugenol	13.642	0.62	0.62
methyleugenol	14.332	5.75	5.75
caryophyllene	14.653	0.24	ND
cis-calamenene	15.426	0.17	ND
germacrene D	15.613	0.31	ND
Total		97.29	96.57

EO: essential oil; NE: nanoemulsion; ND: not detected.

Fuente: Elaboración propia.

Table 2

Comparison of lethal concentrations 50 and 90 (LC_{50} and LC_{90}) of pure essential oil and nanoemulsion of *L. philippiana* against L2 instar larvae of *T. absoluta* by fumigation.

Fumigation Time (h)	Treatment	LC_{50} (95% CL) μL L^{-1} (lower-upper)	LC_{90} (95% CL) μL L^{-1} (lower-upper)	Chi- Square (df)	Slope	p-value
4	EO	27.12 (21.40- 33.89)	75.31 (54.89- 136.78)	15.95 (14)	2.89	0.316
4	NE	4,62 (2.96- 6.20)	27.82 (16.57- 96.12)	8.38 (14)	1.64	0.869
8	EO	14.06 (12.00- 16.43)	30.78 (25.03- 42.01)	13.38 (14)	3.77	0.497
8	NE	7.75 (6.03- 9.37)	15.17 (11.98- 25.70)	20.42 (14)	4.40	0.117

CL: Confidence limit has been calculated with 95% confidence; **df**: degree of freedom.

Fuente: Elaboración propia

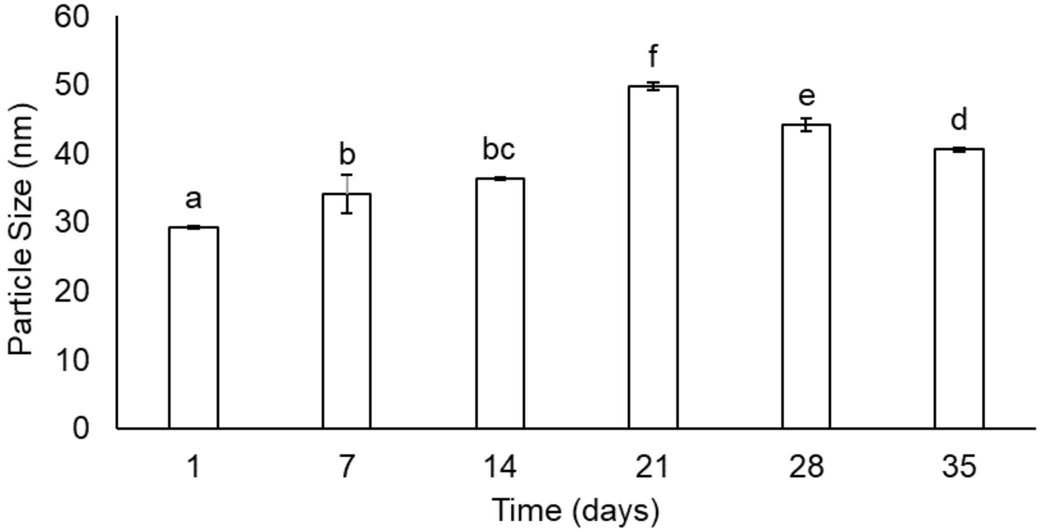

Figure 1: Average particle size (nm) of EO-based nanoemulsion of *L. philippiana* kept at room temperature (evaluated every 7 days over a 35-day period). Analyses were performed using a repeated measures ANOVA, followed by Tukey HSD test ($p < 0.05$). Bars with different letters indicate significant differences in particle size.

Fuente: Elaboración propia.

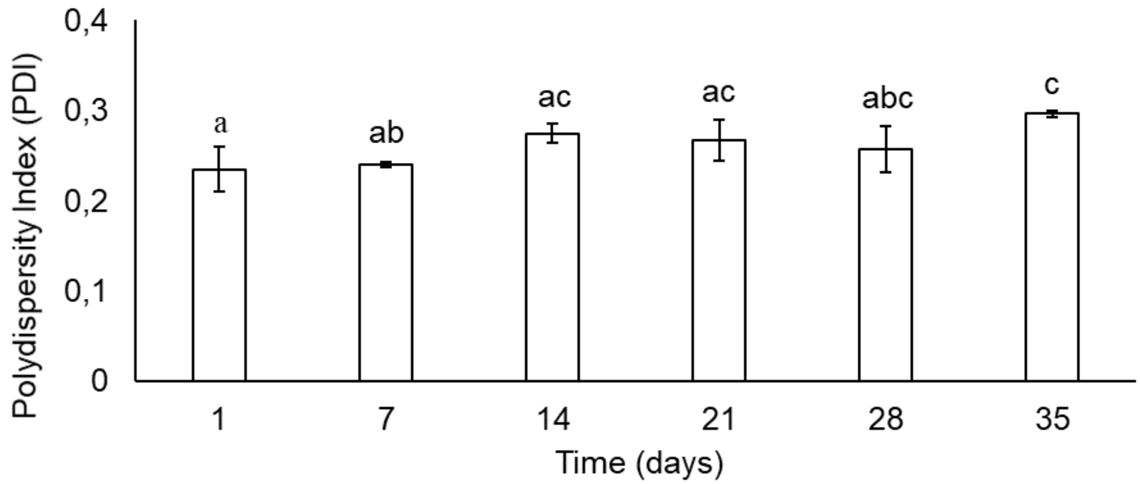

Figure 2: Polydispersion Index (PDI) of EO-based nanoemulsion of *L. philippiana* kept at room temperature (evaluated every 7 days over a 35-day period). Analyses were performed using a repeated measures ANOVA, followed by Tukey HSD test ($p < 0.05$). Bars with different letters indicate significant differences in PDI values.

Fuente: Elaboración propia.

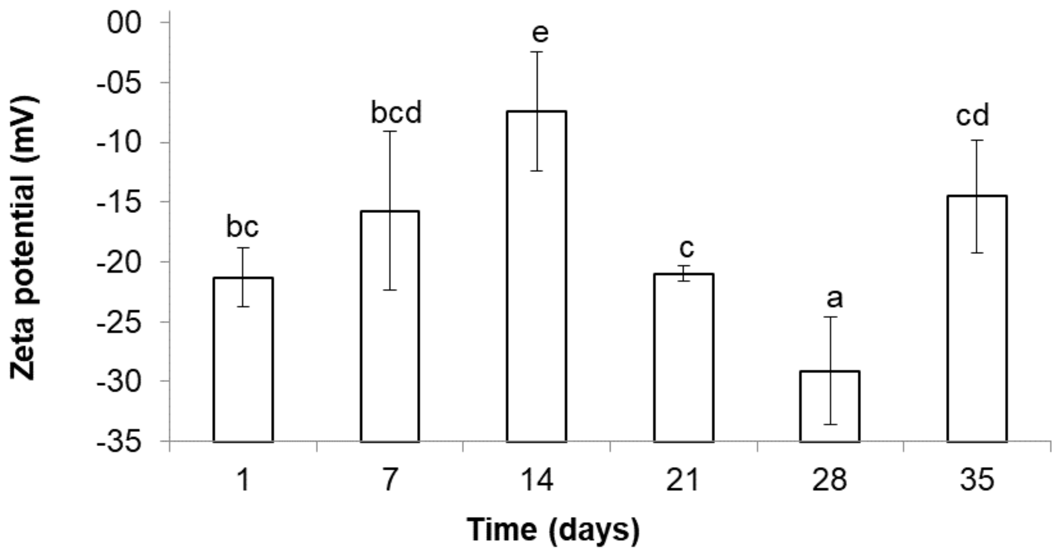

Figure 3: Zeta potential of EO-based nanoemulsion of *L. philippiana* kept at room temperature (evaluated every 7 days over a 35-day period). Analyses were performed using a repeated measures ANOVA, followed by Tukey HSD test ($p < 0.05$). Bars with different letters indicate significant differences in zeta potential.

Fuente: Elaboración propia.

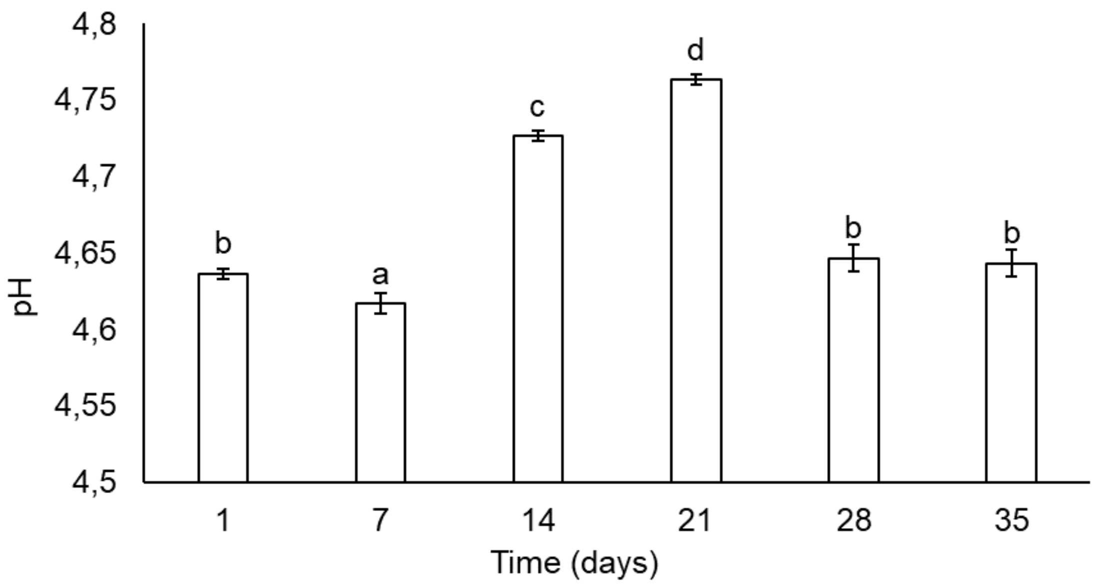

Figure 4: pH of EO-based nanoemulsion of *L. philippiana* kept at room temperature (evaluated every 7 days over a 35-day period). Analyses were performed using a repeated measures ANOVA, followed by Tukey HSD test (p< 0.05). Bars with different letters indicate significant differences in pH.

Fuente: Elaboración propia.

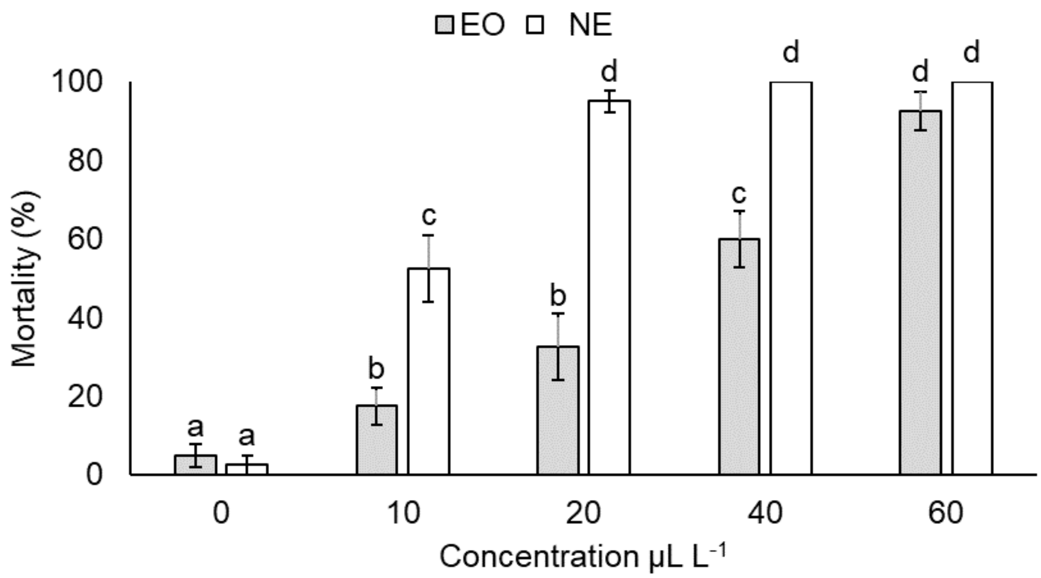

Figure 5. Average mortality rate of L2 instar larvae of *T. absoluta* due to the effect of the pure essential oil and EO-based nanoemulsion of *L. philippiana*, 4 h after fumigation. Significant differences between treatments were estimated by a Factorial ANOVA (Factor 1: Type of formulation (EO and NE); Factor 2: Concentrations (0, 10, 20, 40, 60 µL L^{-1}). Different letters in the bars indicate significant differences between treatments (Tukey HSD test, $p<0.05$).

Fuente: Elaboración propia.

CAPÍTULO 3

CONCLUSIONES GENERALES

Los resultados de este estudio demostraron que el AE de *L. philippiana* tiene un importante efecto insecticida sobre *T. absoluta*. Aún más, al ser formulado como NE puede ser mucho más eficaz contra esta plaga, logrando mayores tasas de mortalidad a concentraciones mucho más bajas que el AE puro.

La NE a base de AE de *L. philippiana* se mantuvo estable durante los 35 días de evaluación, de acuerdo a los parámetros evaluados. El análisis químico mostró que el AE puro y el AE contenido en la NE tienen la misma composición química, con altas concentraciones de linalol, safrol, 1,8-cineol y metileugenol.

Si bien la eficacia de la nanoemulsión a base de AE de *L. philippiana* contra *T. absoluta* fue demostrada, se requiere una mayor estandarización de la NE para que pueda ser una alternativa competitiva a los insecticidas sintéticos. En este sentido, en estudios futuros se necesita evaluar su actividad insecticida contra otras etapas del desarrollo de la plaga, incluyendo bioensayos sobre enemigos naturales y aplicaciones de campo.