UNIVERSIDADE FEDERAL DE LAVRAS

VIVIANA CAROLINA BOJACÁ LÓPEZ

INSECTICIDAL EFFICACY AND NON-TARGET SAFETY OF NANOEMULSIFIED ORANGE ESSENTIAL OIL AND D-LIMONENE: AN ECO-FRIENDLY ALTERNATIVE FOR CONTROLLING *Drosophila suzukii*

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Prof. Dr. Khalid Haddi
Orientador

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EFICÁCIA INSETICIDA E SEGURANÇA PARA ORGANISMOS NÃO ALVO DO ÓLEO ESSENCIAL DE LARANJA E D-LIMONENO NANOEMULSIONADO: UMA ALTERNATIVA ECOLÓGICA PARA O CONTROLE DE *Drosophila suzukii*

Dissertação apresentada à Universidade Federal de Lavras, como parte das exigências do Programa de Pós-Graduação em Biotecnologia Vegetal, área de concentração em Biotecnologia Vegetal, para obtenção do título de Mestre.

APROVADA em 30 de agosto do 2024
Drª. Cláudia Braga, UFVJM
Dr. Juliano Elvis de Oliveira, UFLA

Prof. Dr. Khalid Haddi
Orientador

LAVRAS-MG
2024

Dedico esta dissertação a Deus por iluminar a minha vida todos os dias e sempre manifestar seu infinito amor.

A minha família por estar sempre junto e me ajudar.

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“O mundo não se muda apenas com fatos, embora eles tenham uma importância enorme; o mundo também se muda com ideias. Os gênios e os cientistas ofereceram e oferecem muitas, mas para mudar o mundo como queremos, são necessárias ideias universais, ideias que compreendam e completem as verdades parciais que os grandes nos deixam. É necessária a Ideia, é necessário o Verbo.”

“*Il mondo non si cambia solo coi fatti, anche se essi hanno un'impor-tanza enorme, il mondo si cambia anche colle idee. I geni e gli scienziati ne hanno offerte e ne offrono tante, ma per mutare il mondo come noi vogliamo, occorrono idee universali, idee che comprendano e completino le parziali verità che i grandi ci lasciano. Occorre la Idea, occorre il Verbo.*”

“*The world is not changed by facts alone, even though they are enormously important; the world is also changed by idea. Geniuses and scientists have offered and offer so much; but to change the world as we want, universal ideas are needed, ideas that encompass and complete the partial truths that the great thinkers left us. We need The Idea; we need the Word.*”

(Chiara Lubich)

RESUMO

A crescente preocupação dos efeitos ecotoxicológicos dos pesticidas sintéticos e o desenvolvimento da resistência das pragas a inseticidas têm impulsionado a busca por alternativas mais sustentáveis e ecologicamente corretas, como os óleos essenciais. Estes, devido à sua disponibilidade global, baixa toxicidade para mamíferos e outros organismos não-alvo, bem como custo relativamente acessível, emergem como candidatos promissores para o desenvolvimento de biopesticidas. Neste contexto, a praga *Drosophila suzukii*, conhecida como mosca-da-asa-manchada, representa um desafio significativo para a produção agrícola mundial, atacando uma ampla gama de pequenos frutos e causando danos econômicos consideráveis. Este trabalho investiga uma alternativa ecológica e sustentável, utilizando formulações emulsionadas e nanoemulsionadas de óleo essencial de laranja doce, *Citrus sinensis* e seu principal componente, o limoneno, contra adultos, larvas e pupas de *D. suzukii*. Além disso, foi realizada uma avaliação da toxicidade dessas formulações sobre *Pachycrepoideus vindemmiae*, uma vespa parasitoide com potencial relevante no controle biológico de *D. suzukii*, para avaliar seu impacto em organismos não-alvo. As nanoemulsões de óleo essencial de laranja e limoneno foram preparadas utilizando técnicas de emulsificação de alta e baixa energia, com o objetivo de comparar sua eficácia, otimização da dispersão e estabilidade das partículas. A emulsificação de alta energia resultou na produção de nanoemulsões estáveis, com tamanhos de gotículas na faixa nanométrica (< 450 nm) e baixo índice de polidispersão (PDI < 0,3). Em contraste, as emulsões obtidas por métodos de baixa energia apresentaram menor estabilidade e tamanhos de gotículas maiores. Os testes de toxicidade indicaram que as nanoemulsões produzidas por emulsificação de alta energia apresentaram maior letalidade contra *D. suzukii* em comparação às formulações emulsionadas e às nanoemulsões preparadas por métodos de baixa energia. Quanto à avaliação em *P. vindemmiae*, todas as formulações demonstraram baixa toxicidade. Estes resultados indicam que os óleos essenciais, quando formulados como nanoemulsões, podem ser eficazes no controle de pragas como *D. suzukii*, apresentando uma toxicidade significativa à praga-alvo e oferecendo uma alternativa viável e menos prejudicial ao ambiente e aos organismos não-alvo, em comparação aos métodos convencionais de controle químico. Estes achados são fundamentais para avançar na adoção de estratégias de controle mais sustentáveis, que não apenas combatem eficazmente as pragas, mas também preservam a biodiversidade e a saúde dos ecossistemas agrícolas.

Palavras-chave: Nanoemulsão; óleo essencial; drosófila de asa manchada; controle de pragas.

GENERAL ABSTRACT

The increasing preoccupation with the ecotoxicological problems promoted by synthetic pesticides and pest resistance to insecticides has driven the search for more sustainable and environmentally friendly alternatives, such as essential oils. Consequently, these are attractive candidates for biopesticide development due to their biodisponibility, low toxicity towards mammals and non-target species, and potential affordability. Within this framework, the spotted wing fly, *Drosophila suzukii*, is a significant threat to global agricultural production as an invasive pest of many small and stone fruits and incurs heavy economic damage. This study explores an ecological and sustainable alternative, using emulsified and nanoemulsified formulations of sweet orange essential oil, *Citrus sinensis* and its main component, limonene, against adults, larvae, and pupae of D. *suzukii*. In addition, these formulations' toxicity was evaluated on *Pachycrepoideus vindemmiae*, a parasitoid wasp with relevant potential in the biological control of *D. suzukii*, to assess its impact on non-target organisms. Nanoemulsions of orange essential oil and limonene were prepared using high and low-energy emulsification techniques to compare their effectiveness, dispersion optimization, and particle stability. High-energy emulsification resulted in the production of stable nanoemulsions with droplet sizes in the nanometer range (< 450 nm) and a low polydispersity index (PDI < 0.3). In contrast, the emulsions obtained by low-energy methods showed less stability and larger droplet sizes. The toxicity tests indicated that the nanoemulsions produced by high-energy emulsification showed greater lethality against *D. suzukii* compared to the emulsified formulations and the nanoemulsions prepared by low-energy methods. As for the evaluation of *P. vindemmiae*, all the formulations showed low toxicity. These results indicate that essential oils, when formulated as nanoemulsions, can be effective in controlling pests such as D. suzukii, showing significant toxicity to the target pest and offering a viable alternative that is less harmful to the environment and non-target organisms compared to conventional chemical control methods.

Keywords: Nanoemulsion; essential oils; spotted wing drosophila; control pest.

INDICADORES DE IMPACTO

A busca por alternativas sustentáveis aos pesticidas convencionais é crucial diante dos desafios ambientais, econômicos e sociais da agricultura moderna. Neste contexto, o estudo intitulado “Eficácia Inseticida e Segurança para Organismos Não Alvo do Óleo Essencial de Laranja e D-limoneno Nanoemulsionado: Uma Alternativa Ecológica para o Controle de *Drosophila suzuki*i” avaliou a eficácia inseticida do óleo essencial de laranja (*Citrus sinensis*) e de seu componente ativo, o D-limoneno, em diferentes formas (pura, emulsionada e nanoemulsionada). Os resultados demonstraram que as nanoemulsões são formulações mais ecológicas e eficazes no controle da praga, com alta letalidade para *D. suzukii* e baixo impacto sobre organismos benéficos, posicionando-se como alternativas promissoras aos pesticidas químicos. Além de oferecer uma solução ambientalmente amigável, a pesquisa apresenta impactos concretos e potenciais nas dimensões social, tecnológica, econômica e ambiental. O desenvolvimento de biopesticidas à base de óleos essenciais promove práticas agrícolas mais sustentáveis e acessíveis, beneficiando especialmente comunidades dependentes de cultivos vulneráveis à infestação por *D. suzukii*. Tecnologicamente, o estudo avançou na formulação e caracterização de nanoemulsões, com medições detalhadas realizadas na EMBRAPA, como potencial zeta, tamanho de partículas e viscosidade, garantindo qualidade e aplicabilidade. Os impactos econômicos incluem redução de custos associados ao manejo químico e mitigação de perdas agrícolas, tornando essa alternativa viável para pequenos e grandes produtores. Alinhado aos Objetivos de Desenvolvimento Sustentável (ODS) da ONU, este trabalho contribui diretamente para os ODS 2 (Fome Zero e Agricultura Sustentável), 12 (Consumo e Produção Responsáveis) e 15 (Vida Terrestre). Ao propor tecnologias limpas e inovadoras, reduz a dependência de pesticidas sintéticos, protege a biodiversidade e minimiza danos aos ecossistemas, enquanto promove segurança alimentar e sustentabilidade na produção agrícola. Com relevância ecológica e benefícios ampliados a agricultores e territórios impactados, este estudo reforça a transição para uma agricultura mais equilibrada, ambientalmente responsável e resiliente, com potencial de aplicação global em diversos sistemas agrícolas.

IMPACT INDICATORS

The search for sustainable alternatives to conventional pesticides is crucial in addressing modern agriculture's environmental, economic, and social challenges. In this context, the present study, titled *"*Insecticidal Efficacy and Non-target Safety of Nanoemulsified Orange Essential Oil and D-limonene: An Eco-friendly Alternative for Controlling *Drosophila suzukii"*, evaluated the insecticidal efficacy of orange essential oil (*Citrus sinensis*) and its active component, D-limonene, in different forms (pure, emulsified, and nanoemulsified). The results indicated that nanoemulsions are recognized as more ecological and effective formulations for pest control. They demonstrate high lethality against *D. suzukii* and low impact on beneficial organisms, positioning them as promising alternatives to chemical pesticides. In addition to offering an environmentally friendly solution, the research presents concrete and potential impacts across social, technological, economic, and environmental dimensions. Developing essential oil-based biopesticides promotes more sustainable and accessible agricultural practices, benefiting farming communities reliant on crops vulnerable to *D. suzukii* infestations. From a technological perspective, the study advances the formulation and characterization of nanoemulsions, with detailed measurements conducted at EMBRAPA, such as zeta potential, particle size, and viscosity, ensuring high quality and applicability. The economic impacts include cost reductions associated with chemical management and mitigated production losses, making this alternative viable for both small-scale and large-scale producers. Aligned with Sustainable Development Goals (SDGs), this work directly contributes to SDG 2 (Zero Hunger and Sustainable Agriculture), SDG 12 (Responsible Consumption and Production), and SDG 15 (Life on Land). By introducing clean and innovative technologies, this approach decreases dependence on synthetic pesticides, safeguards biodiversity, reduces ecological harm, enhances food security, and promotes sustainable agricultural practices. With its ecological relevance and extended benefits to farmers and impacted territories, this study reinforces the transition toward a more balanced, environmentally responsible, and resilient agriculture, with potential for global application across diverse farming systems.

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SUMMARY

FIRST PART

1. GENERAL INTRODUCTION

The spotted wing drosophila (SWD), *Drosophila suzukii*, is native to Southeast Asia (T. KANZAWA, 1936). Since its simultaneous detection in Europe (CALABRIA et al., 2012)and the United States (HAUSER, 2011) in 2008, it has spread increasingly throughout Asia, the Americas, Europe, and Africa (KIRSCHBAUM DANIEL S.AND FUNES, 2020), with limited sightings in Oceania (EPPO, 2017).This pest, which affects 64 host species from 25 plant families, has also been observed in Argentina ((BUONOCORE BIANCHERI et al., 2024), Uruguay, Chile (Medina-Muñoz et al., 2015), and Brazil (ANDREAZZA et al, 2017b), where it was first reported in the southern region (BENITO; LOPES-DA-SILVA; DOS SANTOS, 2016) and occasionally in the Brazilian tropical savannah (DEPRÁ et al., 2014).

In the Neotropical region, significant damage has been recorded in various fruit species, including plum (*Prunus sp*.), blackberry (*Rubus sp*.), persimmon (*Diospyros kaki*), cherry (*Prunus sp*.), apricot (*Prunus armeniaca*), raspberry (*Rubus idaeus*), blueberry (*Vaccinium myrtillus*), strawberry (*Fragaria sp*.), and peach (*Prunus persica*) (MENDONCA et al., 2019). Lesser occurrences have also been reported in fig (*Ficus sp*.), kiwi (*Actinidia sp*.), and grape (*Vitis sp*.). Additionally, there are reports of attacks on fruits with more resistant skins, such as orange (*Citrus sp*.) and apple (*Malus* sp.) (LEE ET AL., 2011A; LEE ET AL., 2011B; WALSH ET AL., 2011; ANFORA ET AL., 2012). In the southern region of Rio Grande do Sul, it was found in guava (*Psidium guajava*) and jambolan (*Syzygium jambolanum*), as well as collected in McPhail traps with hydrolyzed protein, installed in peach orchards (SCHLESENER et al., 2015).

The implications of *D. suzukii* extend beyond direct crop damage, influencing numerous facets of horticultural production, particularly in fruit cultivation, necessitating increased investments in pest management. This can substantially burden producers financially. The pest's capability to adapt to new hosts complicates management efforts and increases potential economic losses. The financial impact has been profound in the United States, escalating costs from around $1 million to $15 million for small fruit producers post-invasion (BENITO; LOPES-DA-SILVA; DOS SANTOS, 2016). This alarming situation mirrors in Brazil, where favorable conditions for *D. suzukii* align with major fruit production areas, impacting up to 30% of strawberry and 40–65% of blackberry crops in the south, thereby intensifying the potential for economic detriment (WOLLMANN et al., 2020a). Similar challenges are anticipated as *D. suzukii* spreads across Brazilian territories, with expected rises in pest management expenditures and necessary adaptations to these emerging pest pressures.

Current management strategies for *D. suzukii* heavily depend on chemical controls (GARCIA et al., 2022). However, this reliance on synthetic pesticides raises significant concerns due to their ecological and health implications, notably the toxicity to non-target organisms and the propensity for pests to develop resistance (DEANS; HUTCHISON, 2022; GRESS; ZALOM, 2019a). This backdrop has intensified the exploration of viable natural alternatives, such as biopesticides, which include essential oils known for their potent insecticidal properties, low toxicity to non-target species, and affordable price. In this sense, products of plant origin have gained prominence in research, especially essential oils (EOs), as they are bioactive, biodegradable, and environmentally safe (GIUNTI et al., 2022) , in addition to their raw material availability and good cost-benefit ratio (CAMPOLO et al., 2018). Yet, solubility and stability issues with essential oils have prompted advances in delivery systems, notably nanoemulsions, which enhance the effectiveness of these natural compounds. This study, therefore, assesses the insecticidal efficacy of nanoemulsions derived from sweet orange essential oil and D-limonene against *D. suzukii* and examines their impact on non-target organisms, particularly the parasitoid wasp *Pachycrepoideus vindemmiae*, integrating these findings into a broader Integrated Pest Management (IPM) strategy.

2. THEORETICAL FRAMEWORK

2.1 Overview of *Drosophila suzukii*

Drosophila suzukii (MATSUMURA,1931), commonly referred to as the spotted-wing drosophila (SWD), is a member of the melanogaster species group within the *Sophophora* subgenus (ASPLEN et al., 2015). The melanogaster group is subdivided into various species subgroups, including the suzukii subset, which, along with six other subgroups, comprises the "oriental lineage (STACCONI, 2022). *Drosophila suzukii* is globally recognized as a primary invasive polyphagous pest, exhibiting a preference for hosts characterized by thin epicarps. Among its preferred hosts are strawberry (*Fragaria* spp.) (Rosaceae), blackberry (*Rubus* spp.) (Rosaceae), blueberry (*Vaccinium* spp.) (Ericaceae), and raspberry (*Rubus* spp.) (*Rosaceae*) (WOLLMANN et al., 2020a).

2.1.1 Morphology and life cycle

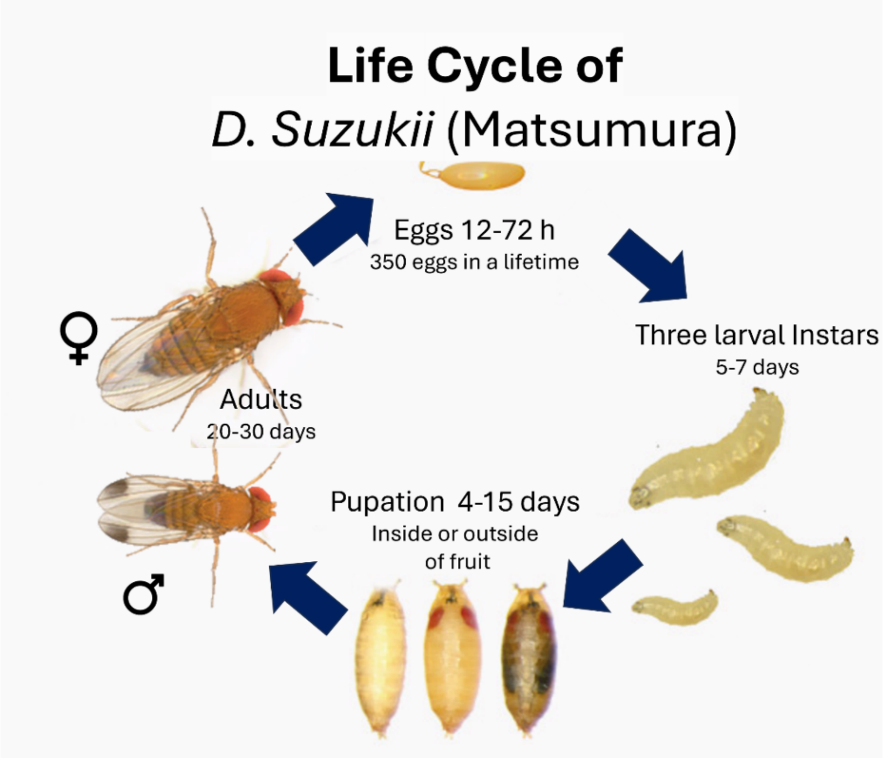
Two distinctive attributes categorize *D. suzukii* as a pest of significant economic risk: its preference for healthy, ripening fruits and the robust, sclerotized, and serrated ovipositor of the female, which pierces the fruit's epidermis, causing material damage (WOLLMANN et al., 2020).The oviposition damage directly affects the fruit and facilitates the entry of secondary organisms that feed on fruits, including other frugivorous insects and pathogenic agents such as fungi and bacteria. This interaction accelerates the decomposition and rotting of the fruit's mesocarp, resulting in considerable production losses with a clear adverse economic impact (Walsh et al., 2011).

The adults measure between 2 and 3 mm, sporting red eyes and a thorax that varies from brown to pale yellow. The males are distinguished by dark transverse bands on their abdomens, a dark spot on each wing, and two sexual combs on their legs. The slightly larger females are characterized by their elongated, serrated ovipositor. The eggs, oval and milky-white, measure about 0.6 mm in length and are equipped with respiratory filaments that aid in respiration within the fruit. Visible by their white color and visible internal organs, the larvae undergo three growth stages, growing up to 5.5 mm in length and significantly contributing to the fruit's decomposition. About 3.5 mm long, the pupae are fusiform and reddish-brown, with spiracles that enable respiration during the pupal phase (RADONJIĆ; HRNČIĆ, 2015).

Moreover, *D. suzukii* can also oviposit in damaged or fermenting fruits, adapting well to fallen fruits when ideal conditions are scarce (TUNGADI et al., 2023). This adaptability allows SWD to thrive under suboptimal conditions, underscoring the need for vigilant and effective pest management to mitigate its adverse (BAL; ADAMS; GRIESHOP, 2017; KIENZLE et al., 2020).

This species has a high reproductive rate (each female can lay more than 350 eggs during its lifetime) and a short life cycle, with an approximate duration from egg hatching to adult emergence of 9-10 days at a temperature of 25°C and 21-25 days at a temperature of 15°C. **Figure 1**.

Figure 1. Life cycle of *D. suzukii*



Source: Cornell University (2023).

2.1.2 Management

To effectively control *D. suzukii*, it is necessary to adopt various strategies to keep the pest population below the economic damage threshold. Thus, Integrated Pest Management (IPM) for SWD assumes the use of different management strategies that are compatible both in the field and after harvest:Monitoring and Trapping: Effective management of *D. suzukii* involves monitoring adult fly populations before fruit ripening and egg-laying begin.

Commonly used bucket-style traps or quart containers, baited with yeast-sugar-water mixtures, fruit purees, or alcohol-based solutions, prove effective for monitoring. Enhancements like adding surfactants or sticky cards improve trap efficacy, particularly under cool and shady conditions, and further improvements to red wine-vinegar attractants included adding specific strains of lactic acid bacteria, *Oenococcus oeni* (ĐUROVIĆ et al., 2021; WALSH et al., 2011).

a) Cultural Control: Maintaining good field sanitation is crucial to preventing the spread of *D. suzukii*. Regular harvesting and removal of damaged fruit reduce the pest population. Cultural control strategies include deep-burying discarded fruits or exposing them to direct sunlight in sealed plastic bags to eliminate insects at various developmental stages (BAL; ADAMS; GRIESHOP, 2017; SCHÖNEBERG et al., 2021).

b) Biological Control: Biological control agents, including parasitoids from families like *Braconidae* and *Figitidae* and predators like *Orius insidiosus* and *Coenosia attenuata*, show promise in controlling *D. suzukii*. Notably, larvae and pupae can be parasitized by species like *Leptopilina heterotoma* and *Pachycrepoideus vindemmiae*. Utilizing *Beauveria bassiana* fungus also offers potential benefits, suggesting synergistic effects when combined with other biocontrol agents (EBEN et al., 2020; LEE et al., 2019).

c) Chemical Control: Despite violations of maximum residue limits for specific pesticides, the development of insecticide resistance and its adverse effects on beneficial arthropods (GARCIA et al., 2022) effective control programs for *D. suzukii* are currently based mainly on chemical methods, with little progress in the use of other compounds to control this pest (see **Table 1**). Effective control programs for *Drosophila suzukii* are mainly based on chemical methods, with few advances in using other compounds to control this pest (see Table 1). In the absence of insecticides specifically registered for *D. suzukii* in the strawberry crop in Brazil, pyrethroids and spinetoram are recommended for emergency use to manage adult populations effectively (BENITO; LOPES-DA-SILVA; DOS SANTOS, 2016).

Table 1. Efficacy of insecticides tested against *D. suzukii* as reported in existing literature.

(Continued)

IRAC MoA Group	Insecticide	Efficacy	Study Reference
Organophosphates(1B) Acetylcholinesterase (AChE) inhibitors Nerve action	Malathion	Excellent	Bruck et al. (2011); Hoffmann Schlesener et al. (2017); Andreazza et al. (2017a, b); Diepenbrock et al. (2016)
	Diazinon	Excellent	Bruck et al. (2011)
Organophosphates(1B) Acetylcholinesterase (AChE) inhibitors Nerve action	Dimethoate	Excellent	Shawer et al. (2018a, b); Hoffmann Schlesener et al. (2017); Andreazza et al. (2017a, b); Profaizer et al. (2015)
	Phosmet	Excellent	Shawer et al. (2018a); Hoffmann Schlesener et al. (2017); Diepenbrock et al. (2016)
	Fenitrothion	Excellent	Hoffmann Schlesener et al. (2017)
	Methidathion	Excellent	Hoffmann Schlesener et al. (2017)
Pyrethroids (3A) Sodium channel modulators Nerve action	Bifenthrin	Excellent	Bruck et al. (2011)
	Beta-cyfuthrin	Excellent	Bruck et al. (2011)
	Permethrin	Excellent	Bruck et al. (2011)
	Zeta-cypermethrin	Excellent	Hoffmann Schlesener et al. (2017); Bruck et al. (2011); Diepenbrock et al. (2016)
	Lambda-cyhalothrin	Excellent	Shawer et al. (2018b); Grassi et al. (2011); Cini et al. (2012); Shaw et al. (2019); Andreazza et al. (2017a, b)
	Deltamethrin	Excellent	Shawer et al. (2018b); Hoffmann Schlesener et al. (2017)
	Fenpropathrin	Excellent	Diepenbrock et al. (2016)
Neonicotinoids (4A) Nicotinic acetylcholine receptor (nAChR) competitive modulators Nerve action	Thiamethoxam	Moderate	Shawer et al. (2018b); Andreazza et al. (2017a, b)
	Thiacloprid	Moderate	Shawer et al. (2018b)
	Acetamiprid	Moderate	Shawer et al. (2018b); Andreazza et al. (2017a, b); Shaw et al. (2019)
	Imidacloprid	Moderate	Shawer et al. (2018b)

Table 1. Efficacy of insecticides tested against *D. suzukii* as reported in existing literature.

(Conclusion)

IRAC MoA Group	Insecticide	Efficacy	Study Reference
Spinosyns (5) Nicotinic acetylcholine receptor (nAChR) allosteric modulators – Site I Nerve action	Spinetoram	Excellent	Beers et al. (2011); Haye et al. (2016); Bruck et al. (2011); Haviland and Beers (2012); Shawer et al. (2018a, b); Shawer (2017); Andreazza et al. (2017a, b); Profaizer et al. (2015)
Diamides (28) Ryanodine receptor modulators Nerve and muscle action	Cyantraniliprole	Excellent	Bruck et al. (2011); Cuthbertson et al. (2014); Beers et al. (2011); Van Timmeren and Isaacs (2013); Shaw et al. (2019); Shawer et al. (2018b); Andreazza et al. (2017a, b)
Pyrolle (13) Uncouplers of oxidative phosphorylation via disruption of the proton gradient Energy metabolism	Chlorfenapyr	Moderate	Andreazza et al. (2017a, b)
Botanical*	Azadirachtin + pyrethrins		
	Monoterpenoids		Monteiro et al. 2021; Pineda et al. 2023; Tong et al. 2013
	Myrtaceae plant EO		Park et al. 2017;Jang et al 2016; Pineda, et al . 2023
	Lamiaceae Plant EO		Park et al. 2016

*Not considered in IRAC MoA classifcation

Source: Author (2024).

2.2 Parasitoid *Pachycrepoideus vindemmiae*

Pachycrepoideus vindemmiae (Rondani) (Hymenoptera: Pteromalidae) is a generalist parasitoid wasp employed in the biological control of the pupal stage of various fly families, including *Drosophilidae, Anthomyiidae, Calliphoridae, Muscidae, Sarcophagidae*, *Tachinidae,* *Tephritidae,* among others (YANG et al., 2019). In Latin America, *P. vindemmiae* has been introduced for classical biological control programs targeting fruit flies in countries such as Argentina (JOOP C et al., 2019), various Caribbean Islands (VAN LENTEREN JC et al., 2020), Costa Rica (BLANCO-METZLER & MORERA-MONTOYA, 2020), Dominica (VAN LENTEREN, 2020), Jamaica (SHERWOOD; LENTEREN, 2020), Mexico (ARREDONDO-BERNAL; RODRÍGUEZ-VÉLEZ, 2020), and Peru (MUJICA; WHU, 2020). In Colombia, *P. vindemmiae* was introduced from the USA and released for fruit fly control in guava plantations from 1984-1985 (LOEHR et al., 2018). The parasitoid is currently mass-produced and marketed in Colombia and is recommended as part of an Integrated Pest Management (IPM) strategy to control *D. inedulis* (BERNHARD LÖHR et al., 2020).Recently, global interest has surged in using *P. vindemmiae* to control the invasive *D. suzukii* (MARIANO-MACEDO et al., 2020; MILLER et al., 2015; ZENGIN; KARACA, 2019).

2.3 Essential Oils

Essential oils, synthesized by plants, are known as secondary metabolites and play crucial roles in plant defense mechanisms and intercellular signaling processes. These compounds play vital roles, functioning as defenses against pathogens and as attractants for pollinators, essential in their reproductive process (ROOHINEJAD et al., 2017). They are synthesized from glucose and accumulate in secretory structures originating from epidermal or parenchymatic tissue. They are distributed across various plant parts, including roots, barks, leaves, flowers, seeds, fruits, and tubers (GIUNTI et al., 2022)

2.3.1 Chemical constituents of Essential oils

Essential oils are phyto-complexes containing between 20 and 60 components in different concentrations. The basic unit of essential oils is called the isoprene unit (C_5H_8; 2-methyl-1,3-butadiene), organized according to the isoprene rule, where the tail is connected to the head of the other isoprene (BUCKLE, 2015). Additionally, essential oils present several attached functional groups that influence their biological activities, the most common being alcohols, ketones, aldehydes, esters, ethers, and phenols. Among terpenes, they are classified according to the number of isoprenes they contain, and can thus be divided into hemiterpenes, monoterpenes, sesquiterpenes, diterpenes, sesquiterpenoids, triterpenes, tetraterpenes, and polyterpenes (MAHAWER et al., 2022). It is common for a single component to constitute more than 20% of the total oil content; for example, d-D-limonene accounts for more than 50% of the components of orange essential oil (BURIANI et al., 2020). Furthermore, essential oils and extracts from certain plants have been studied for their potential as biopesticides, which offer effects comparable to chemical pesticides but with the advantage of being highly degradable in the environment and generally safe for non-target organisms (BENELLI et al., 2017; BURIANI et al., 2020; DUQUE et al., 2023; GIUNTI et al., 2022; SOUZA et al., 2022).

2.3.2 Insecticidal applications

Essential oils in agriculture emerged in the 1990s when their fumigant properties and action as contact insecticides against various pests were discovered (MAHAWER et al., 2022). Due to the high toxicity, negative environmental impact, high cost, and other disadvantages associated with chemical insecticides, essential oils have gained relevance, boosting their research to contribute significantly to the expansion of organic farming, replacing traditional methods.

Recent studies have explored the effectiveness of various essential oils and their components in managing *D. suzukii*. Terpenes and phenylpropanoids have proven effective not only in inducing acute toxicity but also in causing oxidative stress and histopathological alterations in these pests, offering a potentially selective approach for their control (DE SOUZA et al., 2024). Similarly, the essential oil of *Illicium verum* has shown significant toxicity and inhibition of acetylcholinesterase in *D. suzukii*, also causing notable histopathological changes in the insect's tissues (DE SOUZA et al., 2022). Additionally, essential oils from geranium (*Pelargonium graveolens*), dill (*Anethum graveolens*), and Scots pine (*Pinus sylvestris*) have displayed considerable insecticidal and repellent effects against *D. suzukii*. Notably, geranium has demonstrated a deterrent effect, repelling egg-laying females for four days even at the lowest concentration applied (BOŠKOVIĆ et al., 2023). Another study found that essential oils from Several *Baccharis* species, and their main component, D-limonene, exert significant insecticidal effects and deter oviposition in *D. suzukii*. These oils caused adult mortality exceeding 80% at a concentration of 80 mg L^{-1}, with efficacy comparable to that of spinetoram (75 mg L^{-1}). Moreover, D-limonene and the essential oils from *Baccharis* displayed lower LC_{50} and LC_{90} values than those of spinosyn and azadirachtin (DE SOUZA et al., 2021).

Generally, essential oils are recognized for their substantial potential as active ingredients in biopesticides. However, they face challenges related to their physicochemical characteristics; high volatility, limited water solubility, and rapid degradation pose significant barriers to their effective application in practical settings (BENOMARI et al., 2023; HUO et al., 2024; WADHWA et al., 2017). Furthermore, standardizing the chemical composition of essential oils presents difficulties, as samples labeled as identical can vary substantially in their chemical profiles due to factors such as cultivation conditions, extraction methods, and the part of the plant used (BENOMARI et al., 2023; DO et al., 2015).

2.3.3 Mechanism of action

Many essential oils exhibit toxicological properties against insects of the orders Coleoptera, Hemiptera, Diptera, Lepidoptera, Orthoptera Phthiraptera, and Arthropoda Isoptera, acting by various mechanisms, including repellent and antinutritive activity, inhibition of respiration, reduction of growth and fertility, destruction of the cuticle and octopaminergic activity in the central nervous system. (ELISEO et al., 2020).

Recent studies suggest that some of the mechanisms of action of essential oils may be related to neurochemistry and neurohormonality. Prominent among these mechanisms is inhibition of the enzyme acetylcholinesterase (AChE) involved in synaptic transmission (CORRÊA et al., 2023; DUQUE et al., 2023), neurotransmitter regulators such as the γ-aminobutyric acid receptor (GABAaR) and the octopamine receptor (OctpR) (OLIVEIRA et al., 2024; CORRÊA et al., 2023), as well as insect growth regulators (IGRs), which affect the timing of molting during insect metamorphosis, particularly the methoprene-tolerant receptor (MET), which is the physiological receptor for juvenile hormone (JH) (ELISEO et al., 2020; POPESCU; GOSTIN; BLIDAR, 2024).

2.4 Emulsion and nanoemulsion

An emulsion is a colloidal system in which two immiscible liquids, such as oil and water, are mixed with the aid of a surfactant that stabilizes the blend (ELEKAEI BEHJATI; NAVVAB KASHANI; BIGGS, 2017). Depending on which liquid forms the continuous phase that disperses the other, various types of emulsions can be formed: a) oil-in-water (o/w), b) water-in-oil (w/o), and c) multiple emulsions where microdomains of oil and water are dispersed throughout the system(MAHDI; MARAIE, 2019). Nanoemulsions, a subtype of emulsions, are nanometric-sized mixtures that retain these characteristics but with small droplet diameters, typically ranging from 20 to 200 nm (KUMAR et al., 2019; MAHDI; MARAIE, 2019).

Nanoemulsions are formulated by mixing at least three components: oil, water, and an emulsifier. The characteristics and concentration of the main components of a nanoemulsion determine its final properties (SALVIA-TRUJILLO ET AL., 2017). The lipid phase of nanoemulsions can consist entirely of a bioactive lipid, such as an essential oil, or may include a bioactive lipid, such as a vitamin or nutraceutical, dissolved in a carrier oil, such as corn, soybean, sunflower, or olive oil (MCCLEMENTS, 2012). The oily phase can be formulated using different non-polar compounds, such as triglycerides, mineral oils, or essential oils (MCCLEMENTS, 2011; MCCLEMENTS & RAO, 2011). The physicochemical characteristics of the oil, such as viscosity, density, refractive index, and interfacial tension, affect the formation and stability of emulsions and nanoemulsions (VV et al., 2018). Viscosity can influence droplet size; essential oils, which have low viscosity and interfacial tension, can produce smaller droplet sizes than long-chain triglycerides, which have higher viscosity. However, nanoemulsions containing essential oils may have lower long-term stability due to destabilization phenomena such as Ostwald ripening or coalescence (MCCLEMENTS & RAO, 2011). The composition of the aqueous phase plays a vital role in determining the physicochemical properties of nanoemulsions. Various water-soluble constituents, including minerals, acids, bases, flavors, preservatives, vitamins, sugars, surfactants, proteins, and polysaccharides, can be added to the aqueous phase to alter its properties (MCCLEMENTS, 2005). The pH and ionic strength of the aqueous phase influence electrostatic interactions between oil droplets, which can alter the stability of droplet aggregation (QIAN & MCCLEMENTS, 2011). Nanoemulsions are thermodynamically unstable systems and, therefore, require appropriate stabilizers to facilitate the formation of tiny droplets during homogenization and to prevent their aggregation during and after this process (TADROS ET AL., 2004; WOOSTER ET AL., 2008).

2.4.1 Methods of preparation of nanoemulsion

The preparation methods employed in nanoemulsion formulation are critical, significantly influencing their physical stability, bioavailability, and therapeutic efficacy. The methodologies applied are varied and frequently intersect. These techniques are broadly classified into two principal categories: high-energy and low-energy. This classification is based on the energy requirements, the phase inversion type, and the self-emulsification capacity.

2.4.1.1 Low-energy methods

Low-energy nanoemulsion methods leverage the intrinsic physicochemical properties of the system without requiring external energy, making them increasingly popular due to their gentle and non-destructive nature. These methods utilize the system's stored energy to produce nanoemulsions spontaneously as conditions such as temperature and composition are altered, preserving the molecular integrity of encapsulated substances and enhancing process efficiency (ASWATHANARAYAN; VITTAL, 2019). Essential techniques include the Phase Inversion Temperature (PIT) and Phase Inversion Composition (PIC). PIT utilizes temperature-induced changes in non-ionic surfactants to control nanoemulsion droplet size effectively, though it is limited by its reliance on specific surfactant types and potentially high emulsifier use (JIANG; LIAO; CHARCOSSET, 2020; YUKUYAMA et al., 2016). In contrast, PIC involves gradually adding components at a constant temperature, allowing for the formation of tiny droplets, albeit at a slower pace, due to the meticulous addition required (NANTARAT; CHANSAKAOW; LEELAPORNPISID, 2015).

Additionally, spontaneous emulsification and membrane emulsion methods offer unique advantages in nanoemulsion production. Spontaneous Emulsification, which does not require external energy, hinges on surfactant properties and the careful management of cosurfactants like ethanol to form nanoemulsions cost-effectively, although it demands significant quantities of synthetic surfactants (AZMI et al., 2019; BAHUGUNA; RAMALINGAM; KIM, 2020). Meanwhile, Membrane Emulsion produces uniformly sized droplets by pushing the continuous phase through a porous membrane, achieving controlled droplet distribution suitable for various applications but facing challenges at high flow rates and large volumes (DASGUPTA; RANJAN, 2018). Lastly, the Solvent Displacement/Solvent Evaporation method rapidly creates small-sized nanoparticles by emulsifying a polymer solution and evaporating the solvent, offering a fast and efficient process that requires a high solvent-to-oil ratio (AZMI et al., 2019).

2.4.1.2 High-energy methods

High-energy methods involve applying significant fluid stresses to a biphasic liquid mixture. These techniques are used in the food industry because they are easy to use, scalable, reproducible, and high-output. High-pressure homogenization (HPH) involves passing a coarse emulsion through a small orifice under pressures ranging from 500 to 5000 psi to produce very fine droplets, typically less than 500 nm in size. This method is influenced by factors such as the number of passes, homogenization pressure, and phase viscosity ratio, where increasing the number of passes and pressure reduces droplet size (DASGUPTA; RANJAN, 2018; GONÇALVES et al., 2018a).

Microfluidic High-Pressure Homogenization (MFH) channels a coarse emulsion through a high-pressure inlet that splits into smaller branches, causing high shear rates and producing fine nanoemulsions. This process is scalable and widely used for its simplicity in forming fine nanoemulsions with long-term stability (PATHAK, 2017).

In Rotor-Stator Homogenization, an emulsion passes through a narrow gap between a rapidly moving rotor and a stationary stator, effectively reducing droplet size through intense shear and turbulent forces. This method is known for its ability to produce coarse emulsions necessary for further nanoemulsification processes like ultrasonication and is valued for its cost-efficiency and operational simplicity (GAZOLU-RUSANOVA et al., 2020).

Finally, Ultrasonication utilizes high-frequency sound waves to reduce the size of pre-formed emulsions or to create nanoemulsions in situ. This method is particularly noted for its energy efficiency and low surfactant requirement compared to other mechanical methods. Ultrasonication can achieve high droplet stability and minimal coalescence, making it an economically favorable option for laboratory-scale settings, though scaling up poses challenges (MODARRES-GHEISARI et al., 2019; SAFAYA; ROTLIWALA, 2020).

Comprehending the distinct advantages and limitations inherent to both high-energy and low-energy nanoemulsion preparation methods is essential for selecting the most suitable approach according to specific application requirements. **Table 1** provides a comparative analysis of key aspects of these methodologies, offering a concise overview to facilitate informed decision-making in the selection process.

Table 2. Comparison of High-energy and Low-energy Nanoemulsion Preparation Methods.

Aspect	High-energy Methods	Low-energy Methods	Study Reference
Stability	High stability and uniform droplet size due to intense mechanical forces.	Variable stability; dependent on precise control of formulation conditions.	(GONÇALVES et al., 2018b; HADŽIABDIĆ et al., 2017; KOMAIKO; MCCLEMENTS, 2016)
Energy Efficiency	Less energy-efficient; requires mechanical devices that consume significant power.	More energy-efficient; utilizes intrinsic physicochemical properties of system.	(KUMAR et al., 2019; MAHDI; MARAIE, 2019)
Droplet Size	Can achieve very small and uniform droplet sizes.	Droplet sizes can be less uniform and larger.	(ÇINAR, 2017; JAISWAL; DUDHE; SHARMA, 2015; KUMAR et al., 2019)
Component Integrity	May cause degradation of sensitive components due to high energy input.	Gentler on sensitive components as it involves no mechanical stress.	(GONÇALVES et al., 2018b; KUMAR et al., 2019; PEREIRA et al., 2021; WILSON et al., 2022)
Scalability	Scalable but it is essential to address the challenges related to energy consumption and cost.	Easily scalable with minimal energy increase; suitable for large-scale production.	(PEREIRA et al., 2021; PESHKOVSKY; PESHKOVSKY; BYSTRYAK, 2013; SILVA JEREZ; OYARZÚN CAYO, 2021)
Ease of Preparation	Technically demanding; requires sophisticated equipment.	They often require higher concentrations of surfactants to achieve the desired emulsification, but the process is generally simpler and may be less resource-intensive.	(KUMAR et al., 2019; MUSHTAQ et al., 2023; PEREIRA et al., 2021)

Source: Author (2024).

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SECOND PART: ARTICLE

ARTICLE I - Insecticidal Potential of Nanoemulsified Orange Essential Oil and D-limonene: An Eco-friendly Approach for Managing *Drosophila suzukii* and Impact Assessment on Non-target Insects

This article was written following the guidelines of Journal of Pest Science (preliminary version)

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Abstract

The spotted-wing drosophila, *Drosophila suzukii*, a significant pest of small fruits, has prompted the exploration of environmentally friendly alternatives to synthetic pesticides, such as essential oils (EOs). This study evaluates the insecticidal efficacy of orange essential oil and its main component, D-limonene, in their pure, emulsified, and nanoemulsified forms against *D. suzukii*. Utilizing both high-energy and low-energy emulsification methods, we produced nanoemulsions with nanometer-scale droplet sizes (< 450 nm) and low polydispersity indices (PDI < 0.3), which showed enhanced activity compared to other forms. The study also assessed the impact on non-target organisms by testing the toxicity of different formulations to the parasitoid wasp *Pachycrepoideus vindemmiae*. Results indicate that nanoemulsified formulations, particularly those produced via high-energy emulsification, were more effective, offering higher mortality rates against *D. suzukii* pupae and showcasing their potential as viable biopesticides. This research marks the first report of the efficacy of nanoemulsified orange essential oil and D-limonene against *D. suzukii*, presenting them as promising alternatives to conventional pesticides.

Keywords: Spotted wing drosophila, Bioinsecticide, Nanoemulsion, Pest Control.

1 INTRODUCTION

Currently, the world faces new challenges regarding the conservation of natural resources, due to population growth and demand for food. It is estimated that up to 40% of global agricultural production is lost annually due to pests, while plant diseases result in costs of more than US$ 220 billion to the global economy. In addition, invasive insects cause losses of at least US$70 billion (FAO, 2021). In an attempt to avoid these losses and improve productivity, the indiscriminate use of pesticides has increased, leading to the deterioration of soil health (RAJMOHAN; CHANDRASEKARAN; VARJANI, 2020), the degradation of agro-ecosystems, the generation of problems related to waste management, environmental pollution (MITRA et al., 2024) and increased insect and pathogen resistance to pesticides (SHARMA et al., 2019; SIDDIQUI et al., 2023). In addition, the intensive use of pesticides has been associated with adverse effects on human health, such as acute poisoning, cancer, and neurological disorders (ZHOU; LI; ACHAL, 2025).

Given this scenario, it is becoming increasingly relevant to develop innovative and environmentally sustainable approaches to protecting crops and the environment. One promising approach is adopting strategies based on biological and biorational components, i.e. strategies that minimize environmental impact and its effects on ecosystems. In this sense, the convergence between biotechnology and nanotechnology has proved particularly relevant, offering new materials and tools with improved properties compared to conventional methods. Biology, in turn, provides nanotechnology opportunities to explore and apply functional nanostructures inherent to living organisms, expanding the possibilities for more effective and sustainable pest control. The use of natural compounds for pest control is also gaining increasing interest. These compounds, often referred to as biopesticides, include essential oils, which stand out for their wide availability, low toxicity to mammals and non-target organisms, and their relatively affordable cost.

Extracted from various parts of plants, such as roots, stems, and leaves, essential oils are oily, lipophilic, volatile compounds that mainly include terpenes and terpenoids, aliphatic compounds, and aromatic substances (MAHAWER et al., 2022), which have attracted attention for their antimicrobial and insecticidal activity manifested in the form of acute toxicity, repellency, antifeeding, oviposition deterrence, and inhibition of development and reproduction (DURÁN AGUIRRE et al., 2020; ELISEO et al., 2020; POPESCU et al., 2024). Several essential oils, such as those derived from citrus species, are classified as GRAS (Generally Regarded as Safe) by the United States Food and Drug Administration (FDA) due to their favorable safety profiles (MANZUR et al., 2023). Brazil stands out as the largest global producer of citrus fruits, producing 585,448 hectares in May 2022/23 (IBGE, 2024), increasing the availability of EO for use in the agricultural and food industries.

Some recent studies have revealed adult and larvicidal activity in the control of *Culex* mosquitoes of essential oils extracted from citrus fruit seeds (LAGUNDOYE; SIMON-OKE; AKEJU, 2024). Other studies have shown that EOs from the peel of *Citrus* spp. exhibit insecticidal activities against different insects, such as houseflies, mosquitoes, rice weevils, cotton aphids, cartridge caterpillars, and fruit flies (KUMAR et al., 2012; MANZUR et al., 2023; MURUGAN et al., 2012; OYEDEJI et al., 2020; USSEGLIO; DAMBOLENA; ZUNINO, 2023).

However, despite their properties and the great versatility of essential oils, their applications present common restrictions, mainly due to the low solubility in water and the reduced stability of these compounds in aqueous media. With the advance of nanotechnology, nanoemulsions have emerged as a promising strategy to mitigate these limitations, improving the volatility, solubility, and long-term stability of essential oils (POPESCU; GOSTIN; BLIDAR, 2024).

This study compared the insecticidal effect of pure solutions, emulsions, and nanoemulsions of sweet orange essential oil and its main component, D-limonene, against *Drosophila suzukii*, a polyphagous pest known as spotted wing drosophila (SWD). This species, first reported in Brazil in southern subtropical forests (BENITO; LOPES-DA-SILVA; DOS SANTOS, 2016), occurs predominantly in subtropical regions in the south and southeast of the country, causing significant damage to approximately 64 host species, belonging to 25 plant families, of which 60.9% are exotic to the region (GARCIA et al., 2022b). These impacts require greater investment in pest management and control measures, generating additional costs for producers.

In addition, this study assessed the toxicity of nanoemulsions to the non-target organism, the parasitoid wasp *Pachycrepoideus vindemmiae.* This wasp has been used in biological control programs in Integrated Pest Management (IPM), and recent studies have demonstrated its efficiency as a parasitoid of *D. suzukii* under laboratory conditions, indicating its potential for use in programs to increase the population of biological agents (HOGG et al., 2022; LINDER et al., 2022).

2. MATERIAL AND METHODS

2.1 Essential oil and D-limonene specifications

The *Citrus sinensis* essential oil (SCEO) was purchased from WNF essential oils Indústria e Comercio Ltd.a (São Paulo, Brazil). The (R)-(+)-D-limonene 97 %, used for the experimental analysis, was purchased from Sigma-Aldrich (Jurubatuba, SP).

2.2 Fly and Parasitoid rearing

The adults, larvae and pupae of *D. suzukii* were obtained from a stock colony maintained at the Molecular Entomology and Ecotoxicology Laboratory (MEET), Entomology Department, Federal University of Lavras. Flies were housed in transparent plastic cages (V = 700 mL, 4.7 cm in height × 6.7 cm in diameter) sealed with voile mesh under controlled insectary conditions including humidity (60 ± 5%), photoperiod (12:12) and temperature (23 ± 2 °C). They were fed an essential cornmeal diet of cereal flour, corn flour, and water, supplemented with dried yeast. Nipagin was added as an antifungal agent (ANDREAZZA et al., 2016).

Colonies of *P. vindemmiae* were maintained in a climate-controlled chamber at 26±1 °C, with 60±5% relative humidity and a 12 h:12 h photoperiod. These colonies have been sustained in the MEET laboratory and originated from adults that emerged from *Musca domestica* pupae. Adult *P. vindemmiae* were housed in 1.8-liter plastic containers supplied with streaks of pure honey and approximately 100 *M. domestica* pupae, 1 to 2 days old, affixed to a 7x5 cm piece of cardboard using non-toxic white glue, ensuring uniform spacing and secure attachment. The pupae were exposed to the parasitoids for 48 hours under controlled environmental conditions. After this period, the parasitized pupae were carefully removed and transferred to a clean container. Approximately 17 days later, new adult wasps emerged from the pupae.

2.3 Essential oil and D-limonene solutions preparation

Based on preliminary studies conducted by the authors, between five and eight concentration ranges were established for each formulation to determine the lethal concentrations (LC_{50} and LC_{90}) required to achieve 50% and 90% mortality among the insects, respectively. These concentrations were selected as the most promising treatments and subjected to further bioassays.

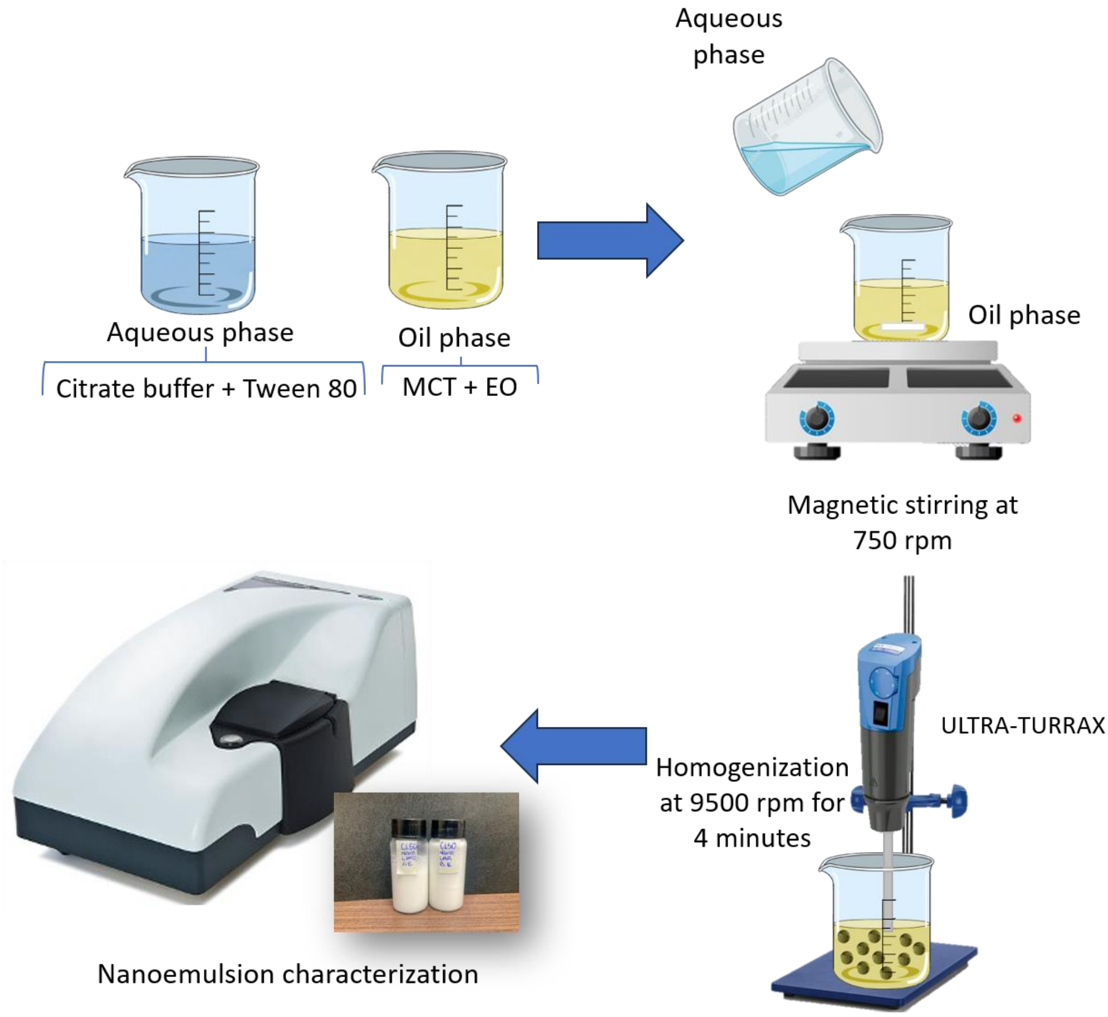
The SCEO solutions were prepared at concentrations of 1, 1.5, 2, 2.5, 2.75, 3, and 3.5 percent by weight (wt%), while D-limonene solutions were prepared at concentrations of 0.25, 0.5, 1, 1.5, and 2 wt%. Each essential oil solution was solubilized in Tween® 80 (0.05 wt%) and distilled water. Prior to pipetting, the solutions were vigorously vortexed for 10 seconds to ensure thorough dispersion of the oil in the water

2.3.1 Preparation of essential oil and D-limonene emulsions

In the development of the emulsion of the SCEO and D-limonene, the oil phase for the emulsion formulation was prepared with varying concentrations of EO: 1.0, 1.25, 2.0, 2. 25, 2.5, 3.5, 4.0, and 5.0 wt%. For D-limonene, the concentrations were 0.5%, 2.0, 2.5, 3. 5, 4, and 5.0 wt%. The surfactant-to-oil ratio was fixed for all emulsions at 3:1. The emulsions were prepared by gradually and continuously adding the EO and surfactant to water, followed by magnetic stirring at 750 rpm.

2.3.2 Low energy nanoemulsions

Nanoemulsions were formed using a method based on spontaneous emulsification, as described by Yuhua Chang et al. (2014), with minor modifications. Standardized conditions for the experiments were as follows: 80 wt% citrate buffer system (5 mM, pH 3.5), 10 wt% Tween 80, and 10 wt% total oil phase. The oil phase was prepared by mixing medium-chain



triglycerides (MCT) with the essential oil at various mass contents: SCEO at 2.5, 2.75, 3, 3.5, 5, and 5.25 wt%, and D-limonene at 1.5, 2.5, 3, 3.5, 5, and 7 wt%. This mixture was then added to an aqueous phase comprising citrate buffer with Tween 80 and stirred magnetically at 750 rpm at ambient temperature. The coarse emulsions were subsequently homogenized at 9500 rpm for 4 minutes using an IKA® T10 basic ULTRA-TURRAX (IKA® SP, Brazil). The samples were stored at 4°C and protected from light (**Figure 1**).

Figure 1. Low-energy preparation of nanoemulsions of SCEO and D-limonene.

2.3.3 High energy nanoemulsion

A two-step process was employed to prepare the O/W nanoemulsions. A courses emulsions were prepared using the same formulation employed for low-energy nanoemulsions as described previously. The oil phase was prepared by combining medium-chain triglycerides (MCT) with essential oil at various concentrations: SCEO at 1.5, 2, 2.5, 3, 3.5, and 5 wt%, and D-limonene at 1.5, 2, 2.5, 3, 3.25, and 3.5 wt%. This mixture was subsequently incorporated into an aqueous phase containing citrate buffer and Tween 80, followed by homogenization at 9500 rpm for 4 minutes using an IKA® T10 basic ULTRA-TURRAX. The courses were then sonicated to obtain emulsions with smaller droplet sizes and a more uniform particle size distribution. A 200W ultrasonic cell crusher Model QR200 (Eco sonics - Ultronique, Sao Paulo, Brazil) with a frequency of 20 kHz was used to ultrasonicate the pre-emulsion for ten minutes at a sonication amplitude of 50% (sonication power: 100 W) as displayed on the device. To control the temperature (and thus isolate its' effects) during the ultrasonication process, all samples were kept in ice-bath. After sonication, the samples were stored at 4°C and protected from light (**Figure 2)**.

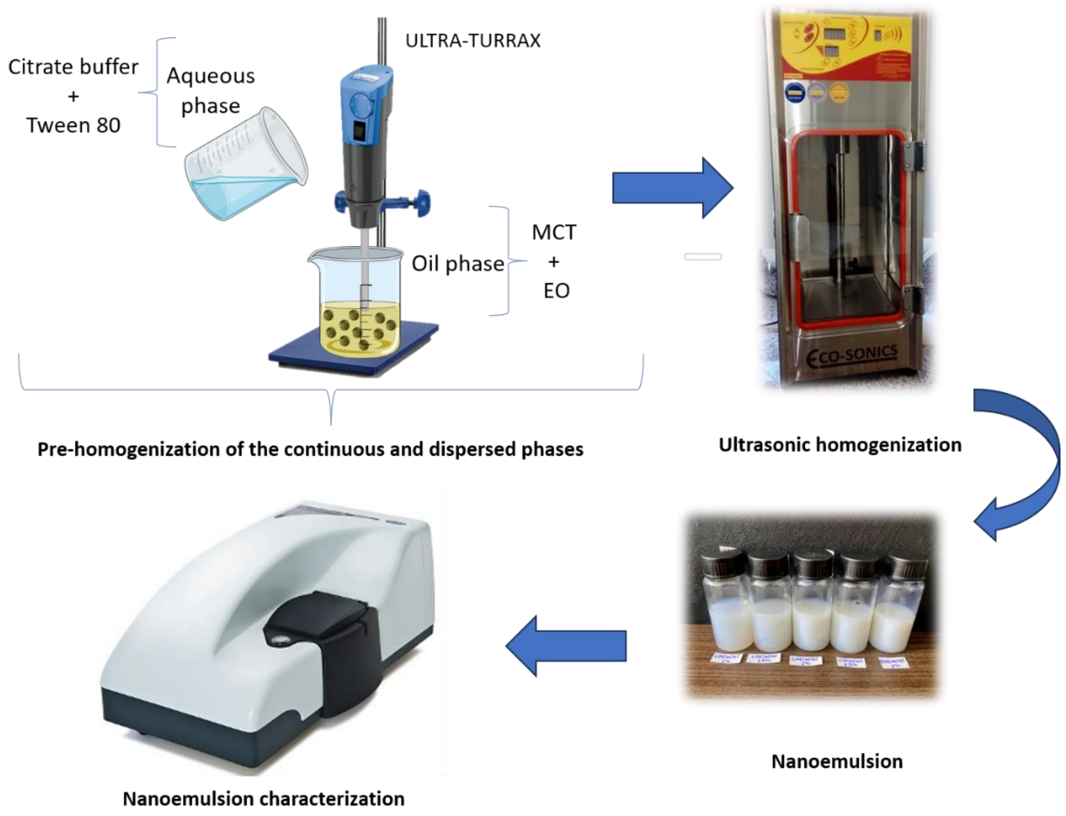


Figure 2. Ultrasound preparation of nanoemulsions of SCE and D-limonene.

2.4 Nanoemulsion characterization

The droplet sizes, polydispersity index (PDI), and zeta potential of CSEO and D-limonene nanoemulsions were measured at 25℃ based on the zeta potential/particle size analyzer of DLS technology using Zetasizer Nano ZS (ZEN3600, Malvern Instruments, Brazil) at the Empresa Brasileira de Pesquisa Agropecuária (EMBRAPA). These measurements were conducted explicitly at the nanoemulsions' lethal concentration levels, CL_{50} and CL_{90}. All samples were tested in triplicate. The viscosity for the prepared nanoemulsion was measured in Brookfield digital Viscometer with the spindle no. 63. Since viscosity could be measured as a function of temperature, the experiment was performed at ambient room temperature 25 °C for all the samples. Furthermore, the prepared nanoemulsions were kept in screw-capped test tubes at 4 °C for seven days to evaluate their physical stability, including assessments of their size, strength, and viscosity.

2.5 Toxicity bioassays with adult flies

The toxicity assessment of SCEO, D-limonene, their emulsions and nanoemulsions against *D. suzukii* followed a bioassay based on the IRAC No. 26 protocol, with minor adjustments (PINEDA et al., 2023). Twenty-five adult *D. suzukii* individuals aged three to five days were introduced into 200 mL glass flasks containing a dental cotton roll (Size #2, 1-1/2" x 3/8") impregnated with 2.2 mL of each treatment. The flasks were sealed with foam plugs to prevent any potential escape of flies. Following the insertion of the emulsion-impregnated cotton roll, 25 non-sexed flies were introduced into individual glass flask (replicate) for 24 hours, following which mortality rates were evaluated. Five to seven concentrations were used for each treatment and all concentrations were replicated (one flask per replicate) four times and they were maintained under controlled conditions (T: 23±2°C, RH: 60±5%, and photophase of 12H).

2.5.1 Toxicity bioassays with larvae and pupae

The essential oil, D-limonene and their nanoformulations were tested to evaluate their larvicidal efficacy against the third instar larvae and pupal satges of *D. suzukii*. The lethal concentrations (LC_{50}) for each preparation (pure, emulsion, and nanoemulsion) were determined from previous tests on adults (**Table 1** in the results section). For the experiment, 10 third-instar larvae were wrapped in voile cloth and gently dipped into the different EO and D-limonene solutions, while those in the control group were dipped in distilled water. Additionally, Tween and buffer were incorporated as additional controls to verify the influence of the solution components on mortality. After being dipped for 10 seconds, the larvae were transferred to a Petri dish containing an artificial diet. Each treatment and the controls were tested in four replicates (n = 4) in addition to control treatments and were transferred to a Petri dish (30 mm in diameter × 15 mm in height).

Larval mortality was assessed at 24 hours by touching each larva with a paintbrush (no. 0), and those not responding were considered dead. The progression of the surviving larvae into pupae and subsequently into adults was monitored up to 10 days after application, considering that the developmental period from egg to adult for *D. suzukii* is approximately 9-10 days at a constant temperature of 25°C (WINKLER et al., 2020).

The experiment was replicated with pupae, utilizing the same procedure, wherein 10 one-day-old pupae were selected. Pupal mortality was subsequently assessed by counting those pupae that failed to emerge as adults within ten days. Individuals displaying visible morphological alterations, such as unsuccessful emergence and deformities in the abdomen, wings, legs, and pronotum, were classified as deformed adults.

2.5.2 Toxicity of CSEO, D-limonene and their various formulations to the parasitoid *Pachycrepoideus vindemmiae*

To determine the toxicity of the CSEO, D-limonene and their nanoformulations to *P. vindemmiae*, the same concentrations (LC_{50} and LC_{90}) used in the larval and pupal bioassays were employed. A volume of 200 µL of each formulation was applied to a piece of Whatman filter paper (No. 1) (20 mm diameter), which was then placed at the bottom of a flat-bottomed glassware tube (height =18.5cm and diameter= 2cm). Ten wasp adults aged three to five days were introduced into each test tube, and the tubes were sealed with foam plugs to prevent escape. Parasitoid mortality was assessed 20 hours after exposure initiation. The experimental design was completely randomized, with 10 replicates per treatment conducted under controlled conditions (temperature: 27±2°C, relative humidity: 60±5%, and a 12-hour photoperiod).

2.5.3 Effect of CSEO, D-limonene and their various formulations on the parasitism of *Pachycrepoideus vindemmiae*

The potential impact of CSEO, D-limonene, and their nanoformulations on the parasitism of the parasitoid wasp *P. vindemmiae* was assessed. Adult wasp females aged 3–4 days were used. Each experimental unit consisted of a glass test tube measuring 2 cm in diameter and

18.5 cm in height, into which 15 *D. suzukii* pupae formed within a maximum of 24 hours. These pupae, previously dipped in each treatment for 10 seconds, were subsequently attached to a 1.5x2.5 cm piece of cardboard using non-toxic white glue. The pupae were exposed to the wasp for 24 hours. Wasp emergence began 17 days after the start of the experiment, and SWD fly emergence was monitored to record non-parasitized pupae during the following five days. Each treatment included ten repetitions, and the bioassay was carried out under controlled laboratory conditions, maintained at 26 ±1 °C, 60 ±5 % humidity, and 12 h:12 h photoperiod.

2.6 Statistical analyses

Lethal concentration (LC_{50} and LC_{90}) and their confidence limits for CSEO and D-limonene were determined by logistic regression in dose-response assays based on the concentration Probit-mortality using in the SAS software (SAS Institute, Cary, NC, USA). Toxicity data for *D. suzukii* larvae and pupae were analyzed using one-way ANOVA to compare the effects of different treatments (essential oils and positive or negative control). Differences between individual treatments were further evaluated using Dunn's post hoc test. Statistical analyses were conducted using R software, version 2022.12. 0..

3. RESULTS

3.1 Characterization studies

The physical characterization of the prepared nanoemulsion was carried out, including assessments of its size, PDI, zeta potential, and viscosity. Measurements were taken explicitly at the emulsions' lethal concentrations CL_{50} and CL_{90}, which were determined by logistic regression in dose-response assays **(Table 1)** of probit mortality. The formulated nanoemulsions exhibited globule size from 200–410 nm and polydispersity ranging from 0.2 to 0.4. The zeta potential values, within the narrow range of -2 to -7, indicate limited stability of the preparations and the viscosity ranging within 2.0–3.5 cP at ambient room temperature **(Table 2)**.

Table 1. Lethal Concentrations (LC_{50} and LC_{90}) of *Citrus sinensis* Essential Oil, D-limonene, and Various Formulations used against Third Instar Larvae and Pupae of *D. suzukii*.

Concentration (wt%)	**Only oil:**		**Emulsion:**		**Nanoemulsion (Low energy)**		**Nanoemulsion (Hight energy)**	
	C. sinensis	Limonene	*C. sinensis*	Limonene	*C. sinensis*	Limonene	*C. sinensis*	Limonene
LC_{50}	1.73 - 1.81	0.54 - 0.59	2.48 - 2.63	2.49 - 2.59	3.38 - 3.49	2.83 - 2.93	1.94 - 2.02	2.11 - 2.18
LC_{90}	2.72 - 2.90	1.11 - 1.25	4.26 - 4.68	3.34 - 3.56	4.34 - 4.85	3.67 - 3.92	2.77 - 2.95	2.84 - 2.97

LC_{50} and LC_{90} are the lethal concentrations that kill 50 % and 90% of tested individuals, respectively. The values presented are given as lower and upper concentration limits, corresponding to the 95 % Fiducial intervals (F.I); $\chi 2$: Chi-square test; P: test probability.

Table 2. Assessment of physical parameters of nanoemulsion

Nanoemulsion Formulation Trial	**Lethal Concentration**	**Average globule Size (nm)**	**Standard Error**	**PDI**	**Zeta Potential (mV)**	**Viscosity (cP) T 25 °C**
C. sinensis Low Energy	**LC_{50}**	796.46	0.075	0.633	2	3.48
	LC_{90}	617.36	0.262	0.548	1.38	3.65
Limonene Low Energy	**LC_{50}**	768.43	0.068	0.644	1.89	3.65
	LC_{90}	693.10	0.258	0.594	1.34	3.72
C. sinensis Hight energy	**LC_{50}**	225.40	0.021	0.217	3.25	2.69
	LC_{90}	289.70	0.025	0.210	6.77	3.68
Limonene Hight energy	**LC_{50}**	221.26	0.008	0.226	3.89	3.27
	LC_{90}	256.33	0.015	0.255	1.922	2.89

3.2 Storage Stability

The physical stability of the prepared nanoemulsions was assessed after seven days storage at 4 °C. The stability evaluation included particle size measurements, zeta potential, and polydispersity index (PDI). The results are summarized in **Table 3**.

Table 3. Physical stability parameters of nanoemulsions after seven days of storage at 4 °C

Nanoemulsion Formulation Trial	Lethal Concentration	Average globule Size (nm)	Standard Error	PDI	Zeta Potential (mV)	Viscosity (cP) T 25 °C
C. sinensis Low Energy	**LC_{50}**	3985	0.462	0.948	1.23	2.79
	LC_{90}	3952	3.291	0.781	0.86	3.09
Limonene Low Energy	**LC_{50}**	3406.76	4.911	1	1.15	3.06
	LC_{90}	1740.10	2.97	0.739	1.08	3.24
C. sinensis Hight energy	**LC_{50}**	289.33	0.057	0.273	2.82	2.05
	LC_{90}	354.56	0.078	0.377	5.90	2.85
Limonene Hight energy	**LC_{50}**	314.90	0.182	0.282	3.27	3.09
	LC_{90}	309.30	0.017	0.236	1.56	2.76

The results of physical stability parameters of nanoemulsions demonstrated an increase in droplet size across all nanoemulsions after seven days of storage at 4°C. For high-energy nanoemulsions, the peak average size escalated from 225.4 nm at preparation to 289.3 nm after seven days for the CL_{50} of *C. sinensis* and from 289.7 nm to 354.6 nm for the CL_{90}. Similarly, the CL_{50} of D-limonene increased from 221.3 nm to 314.9 nm and from 256.3 nm to 309.9 nm for the CL_{90}. Low-energy nanoemulsions exhibited more substantial growth, where the peak average size for the CL_{50} of *C. sinensis* surged from 796.46 nm initially to 3985 nm and from 617.36 nm to 3952 nm for the CL_{90}. For D-limonene, the increases were from 768.43 nm to 3406.76 nm for the CL_{50} and from 693.10 nm to 1740.10 nm for the CL_{90}. The polydispersity index (PDI) and viscosity also showed slight increases, indicating decreased uniformity over time.

The PDI values showed varied results, with some formulations exhibiting an increase, which may imply a broader distribution of globule sizes and potential instability. The zeta potential values remained relatively stable across formulations, indicating that the electrostatic stability of the nanoemulsions was not significantly affected by storage. However, changes in viscosity were observed, with some formulations showing increased viscosity, which could be related to changes in the structural integrity of the emulsions.

3.3 Susceptibility of adult *D. suzukii* exposed to different preparations of CSEO and D-limonene.

The different lethal concentration levels for adult *D. suzukii* after 24 hours were estimated using Probit analysis (Figure 1). The most toxic preparations were pure CSEO (CL_{50}= 1.7 wt%, CL_{90}= 2.72 wt%, χ^2= 2.50, *df* = 5, *P* = 0.77) and pure D-limonene (CL_{50}= 0.54 wt%, CL_{90}= 1.11 wt%, χ^2= 1.76, *df* = 3, *P* = 0.62). These were followed by high-energy nanoemulsion of CSEO (HE. CSEO) with CL_{50}= 1.90 wt%, CL_{90}= 2.77 wt% (χ^2 = 1.4, *df* = 4, *P* = 0.84) and high-energy nanoemulsion of D-limonene (HE. LIM) with CL_{50}= 2.11 wt%, CL_{90}= 2.84 wt% (χ^2 = 1.60, *df* = 4, P = 0.80). The emulsion of CSEO (E. CSEO) showed CL_{50}= 2.48 wt%, CL_{90}= 4.26 wt% (χ^2= 6.0, *df* = 4, *P* = 0.19), and the emulsion of D-limonene (E.LIM) had CL_{50}= 2.49 wt%, CL_{90}= 3.34 wt% (χ^2= 4.6, *df* = 4, P = 0.20). The preparations with the lowest toxicity were the low-energy nanoemulsion of CSEO (LE. CSEO) with CL_{50}=3.38 wt%, CL_{90}=4.56 wt%, (χ^2 = 3.79, *df* = 4 *P* = 0.43) and the low-energy nanoemulsion of D-limonene (LE.LIM) with CL_{50}=2.83 wt%, CL_{90}=3.67 wt% (χ^2 = 3.20, *df* = 3, *P* = 0.36). The control group showed a mortality rate of <2%.

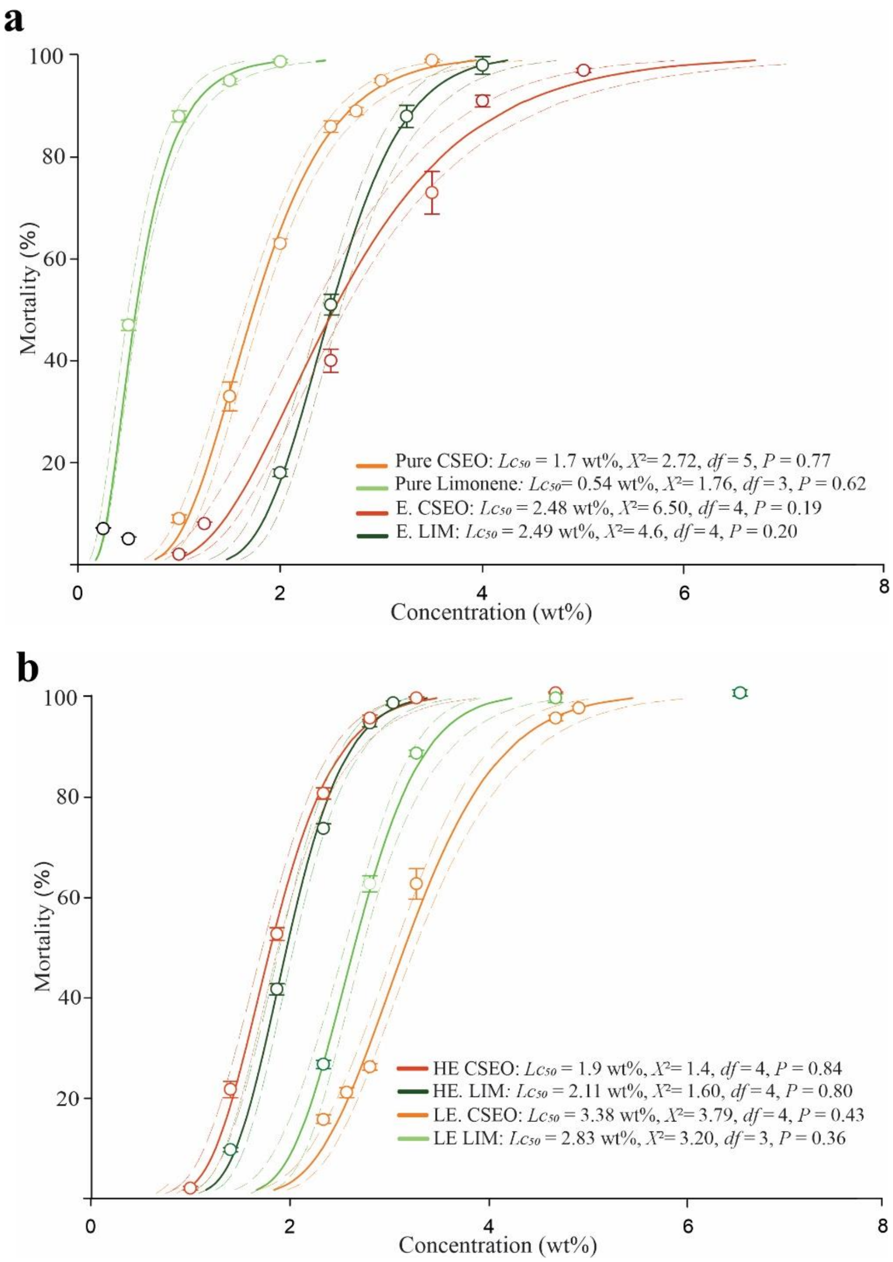


Figure 1. Toxicity of different preparations of CSEO and D-limonene to *D. suzukii* flies. **a)** Preparations with pure CSEO, pure D-limonene, emulsion of CSEO (E. CSEO), and emulsion of D-limonene (E. LIM). **b)** High-energy nanoemulsion of CSEO (HE. CSEO), high-energy nanoemulsion of D-limonene (HE. LIM), low-energy nanoemulsion of CSEO (LE. CSEO), and low-energy nanoemulsion of D-limonene (LE. LIM).

3.4. Toxicity of different preparations of CSEO and D-limonene to *D. suzukii* adults

The adulticidal activity of different preparations of *C. sinensis* essential oil (CSEO) and D-limonene was assessed using the median lethal concentration (CL_{50}) in adults as a reference. The tested formulations included pure CSEO and D-limonene, an emulsion of CSEO (E. CSEO), an emulsion of D-limonene (E. LIM), and a low-energy nanoemulsion of D-limonene (LE. LIM). Additionally, a control group and treatments with Tween and buffer were incorporated as additional controls to verify the influence of the solution components on mortality.

3.5. Toxicity of different preparations of CSEO and D-limonene to *D. suzukii* larvae

Total accumulated mortality, encompassing dead larvae and non-emergent pupae, was evaluated and presented as a percentage of mortality. The mean and standard error for each treatment are depicted in **Figure 2**. Treatments with pure D-limonene and high-energy nanoemulsion of D-limonene (HE. LIM) demonstrated the highest efficacy, achieving a mortality rate of 35%. The low-energy nanoemulsion of CSEO (LE. CSEO) also showed high efficacy, with an average mortality of 32.5%. The high-energy nanoemulsion of CSEO (HE. CSEO) followed closely, exhibiting a mortality rate of 30%. Emulsions of CSEO and D-limonene (E. CSEO and E. LIM) presented average mortalities of 25% and 15%, respectively. Treatments with low-energy nanoemulsion of D-limonene (LE. LIM) and pure CSEO exhibited average mortalities of 20% and 15%, respectively. Conversely, the additional controls with Tween and buffer showed low mortality rates of 2.5% and 7.5%, respectively, similar to those observed in the control group. These findings confirm that the observed toxic effects are primarily attributable to the CSEO and D-limonene rather than the solution's components.

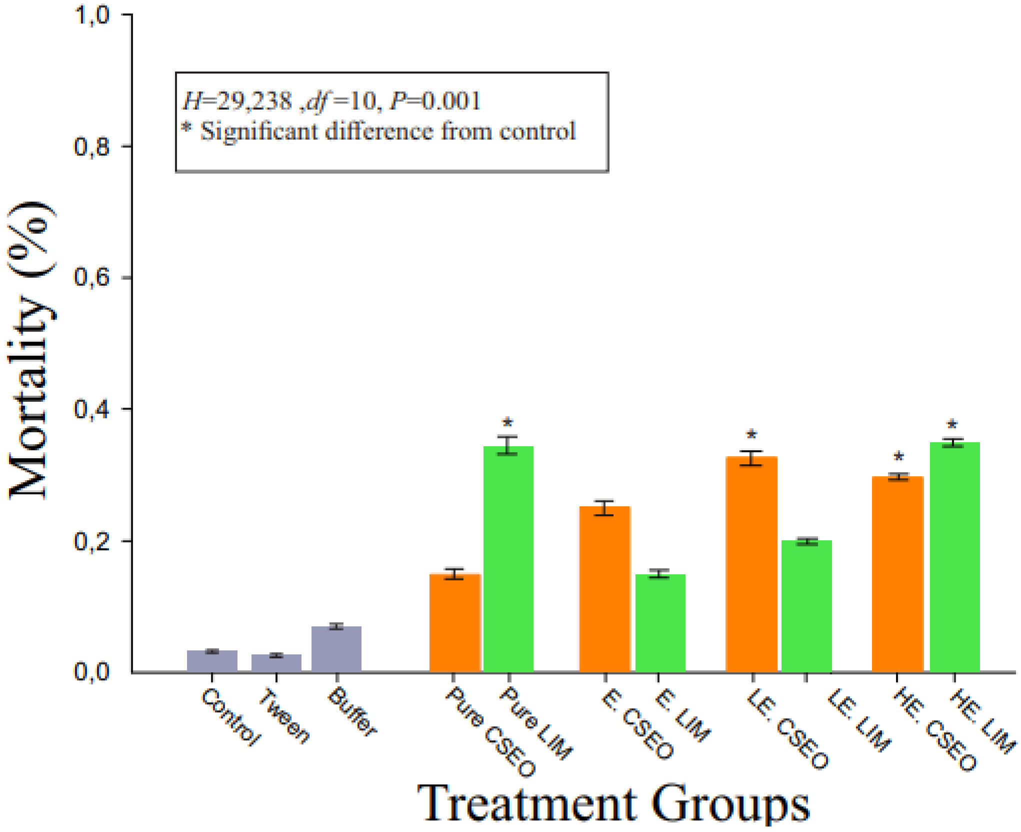


Figure 2. Larvicidal Activity of CSEO and D-limonene Preparations.

3.6 Toxicity of different preparations of CSEO and D-limonene to *D. suzukii* pupae

The analysis of pupal mortality extended our larvicidal research to assess the viability of pupae following treatment exposure, employing the established methodology in which pupae were considered dead if they did not produce live adults. Statistically significant disparities in mortality rates among treatment groups were confirmed through a Kruskal-Wallis test (H=38.014, df = 10, P < 0.001). Notably, the high-energy nanoemulsions of D-limonene (HE. LIM) and CSEO (HE. CSEO) were the most effective, with mortality rates of 85% in both cases (P = 0.001). Other formulations, including low-energy D-limonene nanoemulsion (LE. LIM), Low-energy CSEO (LE. CSEO), and emulsion of D-limonene (E. LIM), also exhibited significantly higher mortality rates, achieving 77.5%, 72% and 75%, respectively. More moderate preparations included CSEO emulsion (E. SCEO), pure D-limonene and pure CSEO essential oil, with 50%, 47.5% and 40% mortality rates, respectively (**Figure 3**).

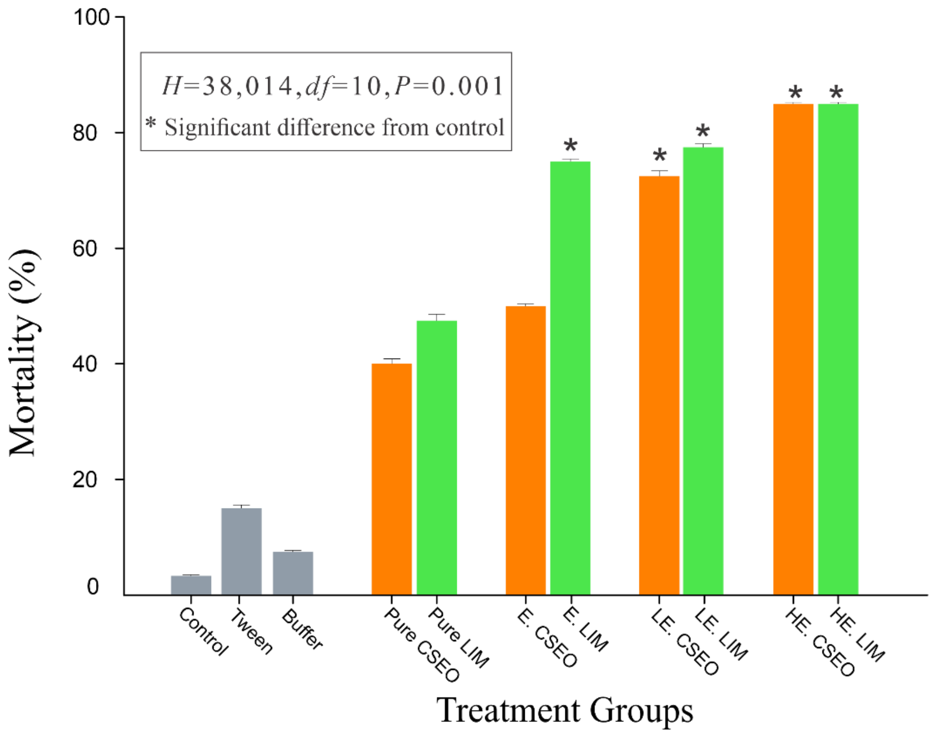


Figure 3. Mortality Rates in Pupae Induced by Various Preparations of CSEO and D-limonene.

In addition to assessing mortality, this study examined the occurrence of morphological malformations in *D. suzukii* pupae after exposure to the treatments, as shown in **Figure 4**. One-way Kruskal-Wallis analysis of variance in ranks indicated significant variations in malformations between treatment groups (H = 25.276, df = 10, P = 0.005), suggesting that the observed differences were not due to chance. In particular, emulsified formulations of CSEO and D-limonene and low-energy nanoemulsion of CSEO (LE. CSEO) showed pronounced effects. E. CSEO and LE. CSEO each showed a 30% incidence of malformations, while E. LIM had an incidence of 27.5%, followed by high-energy nanoemulsion of CSEO (HE. CSEO) with 17.5%. These results were statistically significant for E. LIM (*P* = 0.019), E. CSEO (*P = 0.020*), and LE. CSEO (*P = 0.020*) compared to the control, as confirmed by Dunn's post-hoc comparisons.

Figure 4. Developmental anomalies observed in *D. suzukii* pupae and adults. (**a**) A series of pupae exhibiting unsuccessful emergence. The first two pupae show partial emergence, where the adult flies failed to exit completely, leading to death. The remaining pupae display varying degrees of emergence failure, indicating a potential defect during the emergence process. **(b)** The right side of the image shows a pupa with arrested development, lacking clear morphological differentiation. The left side presents a pupa undergoing normal development, with distinguishable features such as the developing appendages and characteristic pigmentation. **(c)** The image shows two examples of adult flies with severe morphological defects. On the left, a fly with malformed wings is characterized by incomplete extension and abnormal wing morphology. On the right, a fly failed to fully emerge from the pupal case, resulting in incomplete emergence. **(d)** An adult fly exhibited deformed wings post-emergence, a phenotype potentially linked to genetic mutations or environmental stressors during pupation. The wings appear crumpled and non-functional, indicating a defect in the unfolding process during or after emergence.

3.7 Toxicity of different preparations of CSEO and D-limonene on *P. vindemmiae*

As for the toxicity of the different preparations of CSEO and D-limonene on *P. vindemmiae* after 24 hours of exposure, no statistically significant differences were observed among LD_{50}, LD_{90} and negative control (water), except pure SCEO (H = 29,793 df = 18, P = 0.040), which showed a mortality of 20%. Figure 5.

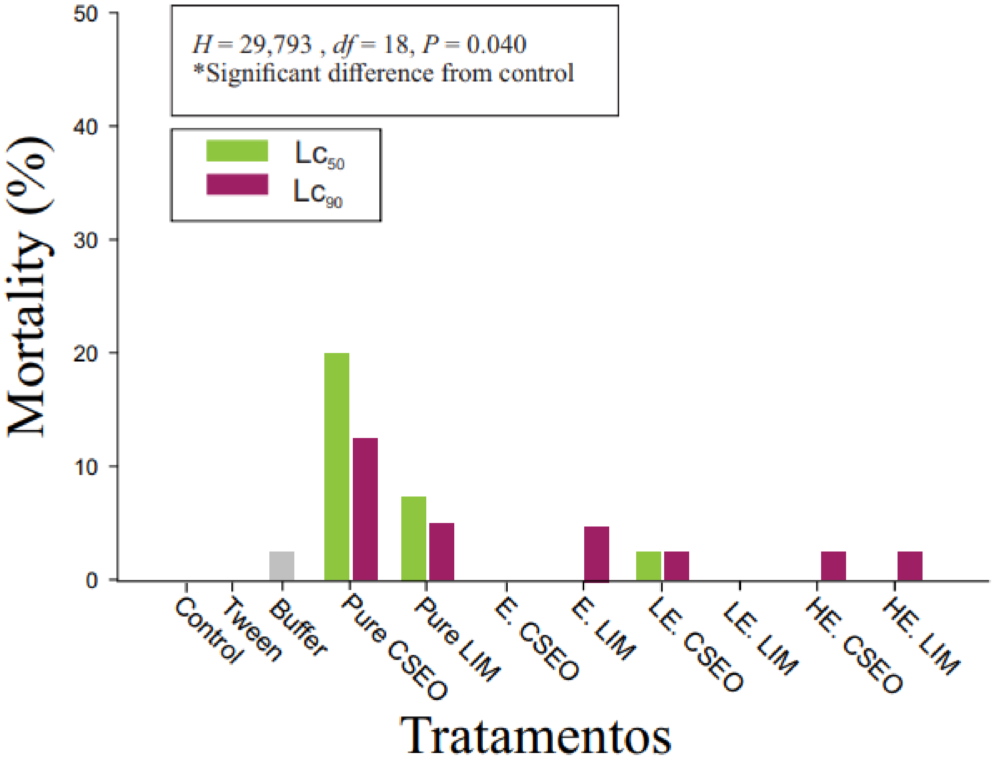


Figure 5. Mortality Rates in *P. vindemmiae* Induced by Various Preparations of CSEO and D-limonene.

3.8 Effect of different preparation on the parasitism of *P. vindemmiae*

The Kruskal-Wallis analysis indicated statistically significant differences between treatment groups (H=118.260, df = 18, P < 0.001), and post hoc comparisons using Dunn's method confirmed that several of the tested formulations resulted in a significant reduction in parasitism compared to the control ($P < 0.05$). Treatments with pure CSEO, pure D-limonene, E. CSEO, and E. LIM at their lethal concentration 50 (LC_{50}) showed a significant difference ($P \leq 0.001$), with parasitism rates of 66%, 68.66%, 72%, and 72.66%, respectively, indicating a considerable decrease compared to the positive control, Tween, and buffer, which achieved parasitism rates exceeding 98%. LE. LIM (P = 0.026) and HE. LIM (P = *0.007*) also exhibited significant differences in parasitism rates, with parasitism rates of 77.33% and 74.66%, respectively, followed by BE. CSEO at 78.66% at the LC_{50} concentration. In contrast, HE.

CSEO demonstrated compatibility with parasitism, with parasitism rates of 91.33% at the LC$_{50}$ concentration.

At the LC$_{90}$ concentration, all treatments showed significant differences ($P < 0.05$) except for LE. LIM and pure D-limonene, with parasitism rates of 78% and 80%, respectively (see **Table 4).**

Nanoemulsion Formulation Trial	Lethal Concentration	Parasitized	Unparasitized and unemerged	Unparasitized and emerged	Parasitism (%)[a]
Negative Control	–	0	6	144	0
Positive Control	–	147	0	3	98.33
Tween	–	148	0	2	98.66
Buffer	–	148	2	–	98.66
pure *C. sinensis*	**LC$_{50}$**	99	48	3	66*
	LC$_{90}$	105	45	–	70*
Pure Limonene	**LC$_{50}$**	103	47	–	68.66*
	LC$_{90}$	120	29	1	80
C. sinensis Emulsion	**LC$_{50}$**	108	42	–	72*
	LC$_{90}$	113	34	3	75.33*
Limonene Emulsion	**LC$_{50}$**	109	41	–	72.66*
	LC$_{90}$	118	30	2	78.66
C. sinensis Low energy	**LC$_{50}$**	118	32	–	78.33
	LC$_{90}$	100	46	4	66.66*
Limonene Low Energy	**LC$_{50}$**	116	34	–	77.33*
	LC$_{90}$	117	33	–	78
C. sinensis Hight energy	**LC$_{50}$**	137	12	1	91.33
	LC$_{90}$	105	44	1	70*
Limonene Hight energy	**LC$_{50}$**	112	38	–	74.66*
	LC$_{90}$	101	45	4	67.33*

a: The parasitism ratio (%) was determined by dividing the mean number of parasitized pupae and pupae placed in the glass test tube(15).

*Significant difference from control , based on Kruskal-Wallis analysis (H=118.260, df = 18, $P < 0.001$), and post hoc Dunn's test ($P < 0.05$)

Table 4. Numbers of *D. suzukii* pupae parasitized or not by *P. vindemmiae*. Parasitized pupae were parasitized by *P. vindemmiae* characterized by the presence of an emergence hole, or pupae parasitized by *P. vindemmiae* but did not result in emergence in these cases, when the larval parasitoid developed a noticeable gap formed between the parasitoid pupa and the puparium shell, making the entire parasitoid pupa visible under a microscope. Unparasitized

and unmerged pupae were fully intact without signs of *D. suzuki*i emergence. Unparasitized and emerged pupal casings showed signs of successful *D. suzukii* emergence.

4. DISCUSSION

The physical characterization of the prepared nanoemulsions revealed significant differences between the high-energy and low-energy methods. The nanoemulsions obtained by high energy showed droplet sizes between 200 and 300 nm, with a polydispersity index (PDI) of less than 0.3, indicating a homogeneous dispersion and potential for physical stability. In contrast, the nanoemulsions produced by low-energy methods exhibited larger particle sizes, ranging between 600 and 800 nm, with a PDI between 0.5 and 0.65. These higher PDI values suggest a wider droplet size distribution and potentially lower stability. This difference was evidenced by measurements taken after seven days of storage at 4°C, which showed a notable increase in droplet size in all formulations, this increase is more pronounced in the nanoemulsions obtained by the low-energy method. This observation is in line with the results of Singh et al. (2022), who observed that a low PDI supports long-term stability, resulting in minimal changes in droplet size, even after two months of storage. In addition, the results indicated that, due to the size of the particles, the emulsions obtained fall more appropriately into the category of microemulsions. This is because the efficiency of producing nanoemulsions can vary significantly depending on the type of surfactant used and the experimental conditions, such as temperature and the synergy between the components of the surfactant/co-surfactant system (SILVA; OYARZÚN, 2021).

A determining factor in the emulsification process is the alteration of the spontaneous curvature of surfactants, which can be achieved using techniques such as Phase Inversion Temperature (PIT) (Singh et al., 2022). For example, the study by Xuan-Tien Le (2022) showed that a cajeput essential oil nanoemulsion produced via PIT remained stable for more than 120 days. In addition, Vinh et al. (2019) successfully formulated black pepper nanoemulsions, showing an average size of 17.9 nm, a low polydispersity index (0.137), and high transparency (OD < 0.05) after one month, also using the phase inversion method, a simple and economical technique. However, we chose to avoid this approach based on increasing the temperature due to the intrinsic volatility of the compounds present in essential oils, which could degrade during the process. However, as far as we know, most of the studies available in the literature do not explore whether low-energy methods, without heating, can adequately preserve the chemical profile of essential oils after the production process.

The zeta potential in the range of -2 to-7 implies low colloidal stability. While these values suggest some electrostatic stability, they are relatively low and it may not be enough for preventing particle aggregation. According to Souza et al. (2023), values further from zero increase the particle repulsion, thus avoiding aggregation and improving emulsification stability. The process of Ostwald ripening, in which the growth of larger droplets at the expense of smaller ones occurs, is a reason for this observed trend of instability conducted on low-energy nanoemulsions, which could lead to a polydispersity increase and potential loss of system stability (REYES; HAMZEHLOU; LEIZA, 2021). These results further emphasize the importance of optimizing the formulation and storage conditions of nanoemulsions to improve their stability along with long-term functionality for industrial use.

The toxicity assessment of various preparations of CSEO and D-limonene on *D. suzukii* highlights the significant influence of the formulation method on insecticidal effectiveness. Although the pure essential oils proved to be more toxic, with lower lethal concentrations, the differences in toxicity between the pure CSEO oil and high-energy nanoemulsions were not substantially large. This indicates that high-energy nanoemulsification is a viable alternative that does not significantly compromise efficacy, given the advantages of nanoemulsions in terms of application and potential long-term stability.

On the other hand, the high toxicity of the CSEO and D-limonene preparations was observed in *D. suzukii* pupae, with the best results obtained using the pure preparations and the high-energy method. This effect can be attributed to the small size of the nanoemulsions, which easily penetrate the insect's cuticle and even individual cells, where they can interfere with physiological processes. In addition, the metabolic processes of pupae differ significantly from those of adults and larvae. During the pupation phase, the Malpighian tubules, essential for rapidly eliminating waste and toxic solutes, stop their physiological activity, as evidenced by the loss of apical microvilli (COHEN et al., 2020). This inactivity can make pupae more susceptible to the action of nanoemulsions.

Furthermore, the results underscore how high-energy methods for preparing nanoemulsions enhance toxicity towards *D. suzukii*. High-energy nanoemulsions showed lower LC_{50} and LC_{90} values compared to low-energy nanoemulsions and the emulsions. This may be due to the production of finer and more uniformly distributed particles, which facilitate better coverage and penetration into the insect tissues, increasing the bioavailability of the active ingredients (GUPTA et al., 2024; HU et al., 2021; ZAHI; LIANG; YUAN, 2015). Li et al. (2012) also managed to produce nanoemulsions with droplet sizes below 100 nm using ultrasonication and a combination of sorbitan trioleate and polyoxyethylene (20) oleyl ether surfactants, demonstrating the efficacy of this method in producing finely dispersed emulsion.

In addition to the toxicity of the various preparations studied on *D. suzukii*, it is also necessary to consider their effect on natural enemies used to manage these pest populations (EBEN et al., 2020; GOWTON; REUT; CARRILLO, 2020; SOUZA et al., 2022). According to our results, all the preparations analyzed caused low mortality in adults of *P. vindemmiae*, making it evident that they are an alternative to Integrated Pest Management (IPM) of *D. suzukii* (EBEN et al., 2020; SOUZA et al., 2022) a significant reduction in parasitism rates was also observed. These results underscore the importance of studying the susceptibility and toxicity between different parasitoid species and essential oils on a case-by-case basis, as these interactions can vary significantly (LOUISE VAN OUDENHOVE et al., 2023; SOMBRA et al., 2022). Therefore, the incompatibility observed in some CSEO and D-limonene formulations with the parasitism rate of *P. vindemmiae* does not necessarily preclude their use in pest control programs, provided they are appropriately integrated with other parasitoids within production systems.

This study has highlighted the complexity and importance of choosing methods for emulsifying and formulating essential oils for insecticide applications. Nanoemulsions prepared by high-energy techniques demonstrated greater efficacy, stability, and uniformity in particle distribution compared to low-energy preparations, which, although less effective, remain a viable alternative, especially in scenarios where cost and simplicity of production are critical factors. The toxicity of the formulations at different stages of *D. suzukii* development, combined with the reduced impacts on natural enemies such as *P. vindemmiae*, indicates that these essential oil nanoformulations are promising and environmentally sustainable. Based on the results obtained, it can be concluded that, once the stability challenges have been overcome, nanoemulsions can be an effective and ecologically appropriate tool for pest management.

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FINAL CONSIDERATIONS

This study shows how essential oil nanoemulsions can be used as a potentially effective pesticide that are both effective and safe for the environment. However, they also highlight important challenges that need to be addressed with respect to their long-term stability, highlighting the need to optimize nanoemulsion formulation, improve surface charge and emulsifier composition to counteract the tendency of nanoemulsions to aggregate.

As for integrated pest management, the results obtained from nanoemulsions are promising for IPM due to their biodegradability and low toxicity to non-allergenic organisms compared to traditional synthetic pesticides, in addition to being able to contribute to improving the effectiveness of pest control and reducing the environmental burden associated with conventional methods, thus contributing to a more sustainable agriculture.

In addition, the need for comprehensive toxicological evaluations in both target and non-target species has been identified to ensure that negative ecological or health effects of nanoemulsions do not outweigh the promised benefits. Nanoemulsion formulations should be tested under a wider variety of environmental and agronomic conditions, as well as in more extensive field trials, to ensure their validation longitudinal studies will be crucial to validate nanoemulsion formulations under a wide range of environmental and agricultural conditions.