ABSTRACT
The PhD thesis focused on the development of eco-friendly essential oil-based nano-pesticides (EO-NPs) for the control of citrus pests such as *Planococcus citri*, *Delottococcus aberiae*, and *Ceratitis capitata*, while also evaluating their effects on non-target organisms. The experiments showed high insecticidal efficacy of the developed nanopesticides against target pests, with low estimated lethal dose values (LD_{50} and LD_{90}). Additionally, a beneficial effect on plants was observed through the overexpression of genes related to salicylic acid (*SA*) and jasmonic acid (*JA*), indicating an enhancement of the natural plant defences. The results showed a variable impact on non-target insects, depending on the route of exposure and insect species. These nanopesticides showed promising potential for use in integrated pest management (IPM) and biological control programmes, combining efficacy and sustainability.

RIASSUNTO
La tesi di dottorato ha avuto come obiettivo lo sviluppo di nanopesticidi eco-compatibili a base di oli essenziali (NPs) per il controllo di parassiti degli agrumi quali *Planococcus citri*, *Delottococcus aberiae* e *Ceratitis capitata*, valutando anche gli effetti su organismi non target. Le sperimentazioni hanno dimostrato un'elevata efficacia insetticida dei nanopesticidi sviluppati contro gli insetti target, con bassi valori delle dosi letali (DL_{50} e DL_{90}) stimate. Inoltre, è stata osservata un'azione benefica sulle piante, testimoniata dalla sovraespressione di geni legati all'acido salicilico (SA) e jasmonico (JA), che indicano un potenziamento delle difese naturali delle piante. I risultati hanno mostrato un impatto variabile sugli insetti non target, in funzione della via di esposizione e delle specie di insetti. Questi nanopesticidi rappresentano una promettente soluzione per programmi di gestione integrata dei parassiti (IPM) e controllo biologico, unendo efficacia e sostenibilità.

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Protection of agro-biodiversity and local productions through the use of new selective biopesticides

Dottorato di Ricerca in Scienze Agrarie, Alimentari e Forestali

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PROTECTION OF AGRO-BIODIVERSITY AND LOCAL PRODUCTIONS THROUGH THE USE OF NEW SELECTIVE BIOPESTICIDES

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In copertina

Agrumeti del sud Italia

Ringraziamenti:
Desidero esprimere la mia più profonda e sincera gratitudine a tutte le persone che hanno contribuito a questo percorso, in particolare al mio tutor, Prof. Vincenzo Palmeri, e al mio co-tutor, Prof. Orlando Campolo. Con il loro supporto, la loro disponibilità e la loro consolidata esperienza nel settore, hanno avuto un ruolo determinante per la realizzazione di questo progetto di dottorato.

Note biografiche
Ho conseguito la laurea magistrale in Scienze e Tecnologie Alimentari (LM-70) presso il Dipartimento di Agraria dell'Università degli Studi Mediterranea di Reggio Calabria e ho completato un dottorato di ricerca in Entomologia Generale e Applicata. Il mio lavoro si concentra principalmente sulla gestione eco-sostenibile degli insetti nocivi. Ho partecipato a progetti di ricerca e pubblicato articoli su riviste internazionali indicizzate.

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SCUOLA DI DOTTORATO
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DIPARTIMENTO
di Agraria

DOTTORATO DI RICERCA
Scienze Agrarie, Alimentari e Forestali

S.S.D. AGRI-05/A
XXXVII CICLO

PROTECTION OF AGRO-BIODIVERSITY AND LOCAL PRODUCTIONS THROUGH THE USE OF NEW SELECTIVE BIOPESTICIDES

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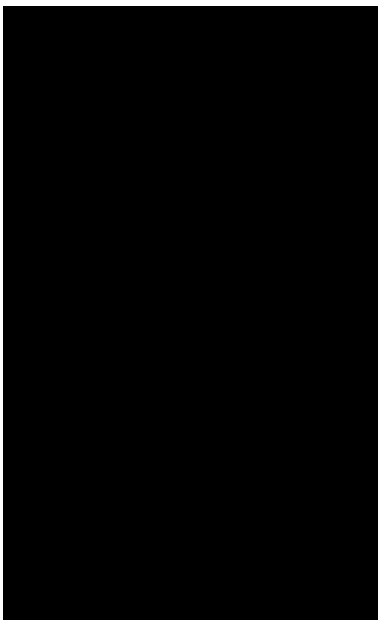


DIPARTIMENTO DI AGRARIA

SUMMARY

Abstract

The huge use of conventional insecticides in crop protection has led to environmental pollution, negative effects on human health and non-target organisms (i.e. useful insects, plants, aquatic organisms, mammals, invertebrates, etc.), and increased insect resistance phenomena that limit the efficacy of control treatments. For these reasons, in the last decade, the development and use of alternative control tools to conventional pesticides have had a growing interest. Among the alternatives, botanical extracts, in particular essential oils (EOs), seem to be ideal candidates for use as bioinsecticides due to certain characteristics (i.e. wide distribution, low cost, insecticidal activity, attractiveness to pollinators, presumed safety towards natural antagonists, etc.). On the other hand, these natural substances have some negative properties (i.e. flammability, volatility, rapid degradation, phytotoxicity, poor solubility in water) which limit or prevent their use in real-world conditions. In recent years, the use of nanotechnology as a tool for developing EO-based nanopesticides (EO-NPs) attracted the interest of different stakeholders. The advantage of using nanotechnology lies in the possibility of mitigating the typical disadvantages of these extracts and improving the chemical, physical and biological properties of their insecticide formulations. In this context, the proposed PhD thesis aimed at the development of new and eco-friendly EO-NPs and to investigate their biological activity against some key citrus pests such as *Planococcus citri* Risso and *Delottococcus aberiae* De Lotto (Hemiptera: Pseudococcidae) and *Ceratitis capitata* (Wiedemann) (Diptera: Tephritidae). The undesired effects towards different non-target organisms (natural enemies, pollinators and plants) were also investigated. To achieve the aim of the thesis, firstly, the chemical profiles of the EOs were evaluated using a gas chromatography/mass spectrometry (GC-MS) apparatus. Then EO-NPs were developed through mixed bottom-up and top-down processes in order to assess the best production method. The developed nano-emulsions were physically characterised through Dynamic Light Scattering (DLS) analysis and particle size, polydispersity index (PDI), surface charge and stability over time were estimated. The most promising EO-NPs were tested against target insects and lethal doses (LD_{50} and LD_{90}) were estimated. To investigate the insecticidal efficacy and the mode of action of the developed formulations, the estimated LDs were used in different bioassays involving both target and non-target species. Further analyses were carried out on plants to assess the phytotoxic effects and the influence on different genes involved in the salicylic acid (SA) and jasmonic acid (JA) pathways. Overall, the research activities allowed to identify the best method for obtaining stable EO-NPs and proved the high insecticidal activity of these nanoformulations against target insects.

In addition, the overexpression in *SA* and *JA* genes underlines a dual benefit action (direct pest control - enhance plant defences) of developed EO-NPs. The results towards non-target insects highlighted the safety of the EO-NPs in indirect and topical treatments while a high mortality rate was registered when applied by ingestion route. The obtained results highlighted the potential of these promising formulations for their use in IPM or biological control programs.

Keywords: sustainable agriculture, essential oils, nanotechnology, citrus pest, non-target organisms

Riassunto

L'uso massiccio degli insetticidi convenzionali per la protezione delle colture ha portato all'inquinamento ambientale, a effetti negativi sulla salute umana e sugli organismi non bersaglio (es. insetti utili, piante, organismi acquatici, mammiferi, invertebrati, ecc.). Per questi motivi, nell'ultimo decennio lo sviluppo e l'uso di strumenti di controllo alternativi ai pesticidi convenzionali hanno suscitato un crescente interesse. Tra le alternative, gli estratti botanici, in particolare gli oli essenziali (OE), sembrano essere i candidati ideali per essere utilizzati come bioinsetticidi grazie ad alcune caratteristiche (es. ampia distribuzione, basso costo, attività insetticida, attrattiva per gli impollinatori, presunta sicurezza nei confronti degli antagonisti naturali, ecc.) D'altra parte, queste sostanze naturali hanno alcune proprietà negative (es. infiammabilità, volatilità, rapida degradazione, fitotossicità, scarsa solubilità in acqua), che ne limitano o impediscono l'uso nelle reali condizioni operative. Negli ultimi anni, l'uso delle nanotecnologie come strumento per lo sviluppo di nanopesticidi a base di OE ha suscitato l'interesse delle diverse parti interessate. Il principale vantaggio delle nanotecnologie risiede nella capacità di mitigare gli svantaggi tipici di questi estratti e di ottimizzare le proprietà chimiche, fisiche e biologiche delle relative formulazioni insetticide. In questo contesto, la tesi di dottorato proposta ha avuto come obiettivo lo sviluppo di nuove formulazioni ecocompatibili a base di OE e lo studio della loro attività biologica contro alcuni parassiti chiave degli agrumi come *Planococcus citri* Risso, *Delottococcus aberiae* De Lotto (Hemiptera: Pseudococcidae) e *Ceratitis capitata* (Wiedemann) (Diptera: Tephritidae). Inoltre, sono stati studiati anche gli effetti indesiderati nei confronti di diversi organismi non bersaglio (nemici naturali, impollinatori e piante). Per raggiungere l'obiettivo della tesi, in primo luogo sono stati valutati i profili chimici degli OE utilizzando lo strumento gas-cromatografo/spettrometro di massa (GC-MS). Successivamente, sono state sviluppate le formulazioni attraverso processi misti *bottom-up* e *top-down*, al fine di valutare il metodo di produzione migliore. Le nano-emulsioni sviluppate sono state caratterizzate fisicamente mediante analisi di Diffusione Dinamica della Luce (DLS), e sono state stimate le dimensioni delle particelle, l'indice di polidispersione (PDI), la carica superficiale e la stabilità nel tempo. Le formulazioni più promettenti sono state testate contro gli insetti bersaglio e sono state stimate le dosi letali (DL_{50} e DL_{90}). Per studiare l'efficacia insetticida e le modalità di azione delle formulazioni sviluppate, le DL stimate sono state utilizzate in diversi biotest che hanno coinvolto specie bersaglio e non bersaglio. Ulteriori analisi sono state condotte sulle piante per valutare i potenziali effetti di fitotossicità e l'influenza su diversi geni coinvolti nelle vie dell'acido salicilico (*SA*) e dell'acido jasmonico (*JA*). Nel complesso, le attività di ricerca hanno

permesso di identificare il metodo migliore per ottenere formulazioni stabili, e hanno dimostrato l'elevata attività insetticida di queste nanoformulazioni contro gli insetti bersaglio. Inoltre, la sovraespressione dei geni *SA* e *JA* ha sottolineato un duplice effetto delle formulazioni sviluppate (controllo diretto dei parassiti - potenziamento delle difese delle piante). I risultati sugli insetti non bersaglio hanno dimostrato la sicurezza delle formulazioni nei trattamenti indiretti e topici, mentre hanno evidenziato un alto tasso di mortalità quando sono stati applicati per ingestione. I risultati ottenuti evidenziano le potenzialità di queste promettenti formulazioni per il loro impiego nei programmi di lotta biologica o di gestione integrata dei parassiti (IPM).

Parole chiave: agricoltura sostenibile, oli essenziali; nanotecnologia; parassiti degli agrumi, organismi non bersaglio

CHAPTER 1

General Introduction

1.1 Citrus pests

Citrus crops are among the most important agricultural products worldwide due to their high economic and nutritional value, with more than 10 million hectares cultivated (FAOSTAT, 2022). However, these crops have several key insect pests that can significantly affect yield and fruit quality. Among the most devastating pests, *Planococcus citri* Risso, *Delottococcus aberiae* De Lotto (Hemiptera: Pseudococcidae), and *Ceratitis capitata* (Wiedemann) (Diptera: Tephritidae) are considered key pest species in many Countries (Urbaneja et al. 2020). The damage caused by these insects can be divided into direct and indirect damages. Direct damages are a consequence of their feeding activity, which weakens the plant by extracting nutrients, resulting in chlorosis, defoliation and stunted growth. For instance, *P. citri* and *D. aberiae* feed at the expense of plant fluids inducing fruit deformation and a reduction in the size. *Delettococcus aberiae*, in particular, has been observed to produce severe irregularities in fruit growth, which drastically reduces the market value of the affected fruits (Pérez-Rodríguez, J. 2020). Indirect damage is typically associated with the secretion of honeydew, which facilitates the proliferation of sooty mould that covers the fruits reducing market values, and the transmission of pathogens.

Ceratitis capitata commonly known as the Mediterranean fruit fly, lays eggs within the fruit, often resulting in premature drop of the fruit (Giunti et al. 2023).

In general, the management of these pests is mainly based on the use of conventional pesticides that include organophosphates, carbamates, pyrethroids, and neonicotinoids (Urbaneja et al. 2020). However, although these substances are efficacy in managing these pests, their huge use, coupled with poor biodegradability, has led to the accumulation of active ingredients (a.i.) in the environment, resulting in contamination of soils, groundwaters and oceans (Ngegba et al. 2022). Furthermore, these broad-spectrum pesticides are reported to be disruptive to beneficial species such as pollinators, natural enemies, invertebrate communities, mammals, aquatic organisms, plants etc. In addition, the increasing resistance of the target insects to chemical treatments has decreased the long-term efficacy of these substances (Mota-Sanchez et al. 2002; Whalon et al. 2008). The growing awareness of the adverse effects of these substances, able to negatively affect the agro-biodiversity, and the introduction of increasingly stringent regulations on their use are prompting stakeholders to identify and develop new, eco-friendly pest management tools.

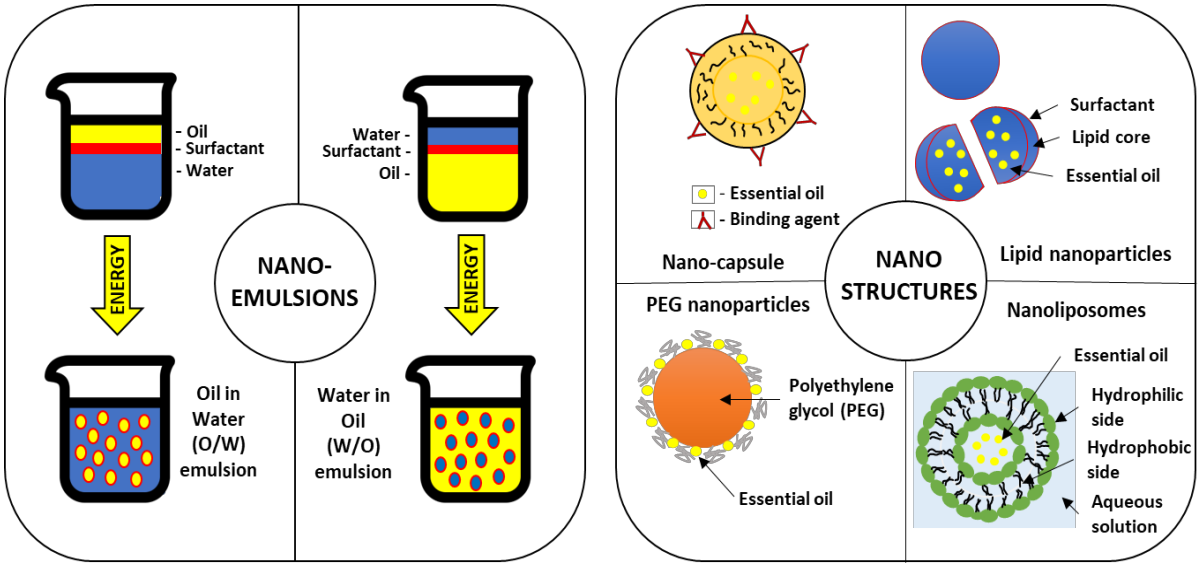
1.2 Botanicals as natural pesticides

Botanicals (extracts, powders, hydrolates and EOs) are natural substances extracted from different botanical families (i.e. Asteraceae, Cupressaceae, Lamiaceae, Lauraceae, Rutaceae, Myrtaceae, etc.) (Svoboda and Greenaway 2003). Botanicals are considered blends of a wide variety of secondary metabolites (i.e. aldehydes, alcohols, monoterpenes; sesquiterpenes; phenylpropenes, esters, phenols etc.) that are synthesised from endocrine and exocrine glands and epidermal cells and are accumulated in different parts of the plant (i.e. leaves, flowers, branches, roots, seeds, fruits) (Campolo 2022). These substances are known to be used by plants in response to different abiotic and biotic stresses (Guleria and Tiku 2009). Botanicals are considered ideal candidates for use as biopesticides due to some characteristics such as proven insecticidal activity, safety to mammals, presumed low toxicity to non-target organisms (i.e. natural antagonists, pollinators, invertebrates, aquatic organisms, plants, etc.), multisite activity, biodegradability of a.i., wide availability and relatively low cost (Regnault-Roger et al. 2012; Giunti et al. 2022). In this context, the biological activity of botanicals against several pests of crops and stored products, as well as blood-feeding insects, has been well investigated (Shaalan et al. 2005; Hikal et al. 2017; Campolo et al. 2018; Lengai et al. 2020). The efficacy of botanicals can be attributed to their ability to affect biological, physiological and metabolic traits (Franzios et al. 1997; Priestley et al. 2003; Zhou et al. 2008). As an example, Wang et al. (2024) investigated the biological activity of seven different EOs against *A. gossypii* highlighting the antifeedant effect and reduction in adult longevity and fecundity. Similarly, Benelli et al. (2012) verified the ingestion, topical and fumigation efficacy of various EOs against *C. capitata*. Instead, other authors highlighted the toxic and repellent activity against Pseudococcidae species (Erdemir and Erler 2017; Elhosieny Mostafa et al. 2018; Alloui-Griza et al. 2022; Mwanauta et al. 2023). Despite the high efficacy of botanicals as insecticides, few formulations are available for use under real field conditions due to some negative properties of botanicals, including: i) chemical instability; ii) flammability; iii) photo-lability; iv) rapid evaporation of a.i.; v) phytotoxicity; vi) poor solubility in water (Pavela and Benelli 2016; Karalija et al. 2020). These challenges are key aspects that need to be overcome to improve the commercial availability of these natural products.

1.3 Nanotechnology in pest management

Nanotechnology refers to all the techniques that enable to obtain materials with physical properties, particularly the size of the particles, ranging in the nanoscale (<100nm). However, when applied to nano-pesticide production, nanotechnology refers to nano-delivery systems with particle sizes lower than <1000nm (Koul 2019; Modafferi et al. 2024a). The advantages of using nanotechnology, combined with the efficacy of the botanicals as insecticides, allow the development of new and eco-friendly nano-tools with unique chemical, physical and biological properties: i) greater solubility in water; ii) improved surface coverage; ii) slow release of a.i.; iv) slow degradability of the formulation; v) reduced phytotoxicity; vi) improved permeability through the insect cuticle; vii) improved bioactivity (Pavoni et al. 2020; Campolo 2022). Botanical-based nanoformulations developed to be used as insecticides can be classified into two main categories such as nano-emulsions and nanostructures (Fig. 1) (Campolo et al. 2017; Modafferi et al. 2024a).

Figure 1: Main types of nano-carriers used to formulate botanical-based bioinsecticides. Modafferi et al. (2023) modified.



1.3.1 Nano-emulsions

These nanoformulations are dispersed systems consisting of a mixture of two immiscible liquids (such as water and oil) that are stabilised through emulsifying substances (such as surfactants) (Campolo et al. 2020). The nano-emulsions can be divided into two main categories, oil in water (o/w) or water in oil (w/o), depending on the composition of the continuous phase, if oil or water respectively. Due to their well-known fabrication procedures, nano-emulsions are considered one

of the best ways to develop insecticide formulations (Campolo 2022). These nanoformulations can affect the target pests through different routes of exposure such as ingestion, contact toxicity, and fumigation (Benelli 2018; Bidyarani et al. 2023). Taking into consideration the different crop pests, the biological activity of botanical-based nano-emulsions was well-proven (Melanie et al. 2022). *Allium sativum* EO-based nano-emulsions were efficacy against *Planococcus citri* Risso (Hemiptera: Pseudococcidae) (Modafferi et al. 2024c, b). Similarly, Laudani et al. (2022) highlighted the efficacy of a *Citrus sinensis* EO-based nano-emulsion against *Aphis gossypii* Glover (Hemiptera: Aphididae). Concerning the stored product insects, several authors reported that different EO-based nano-emulsions were efficacy against *Tribolium confusum* Du Val and *Tribolium castaneum* Herbst (Coloptera: Tenebrionidae), *Cryptolestes ferrugineus* (Stephens) (Coleoptera: Cucujidae), *Rhyzopertha dominica* (F.) (Coleoptera: Bostrichidae) and *Sitophilus oryzae* L. (Coleoptera: Curculionidae) (Golden et al. 2018; Giunti et al. 2019, 2021; Palermo et al. 2021; Draz et al. 2022). In addition, the biological activity of these botanical-based nano-insecticides against blood-feeding insects was also confirmed (Echeverría and Albuquerque 2019; Esmaili et al. 2021).

1.3.2 Nanostructures

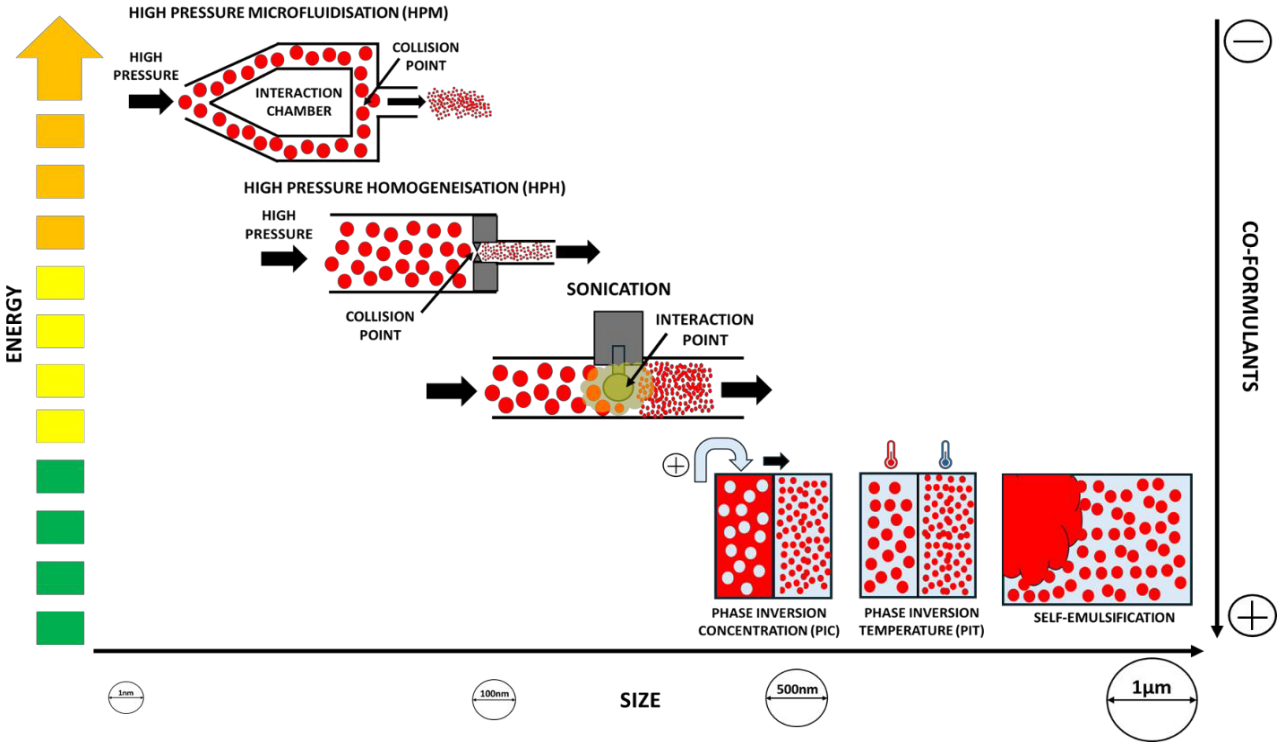
Nanostructures or nanoparticles are solid or liquid formulations developed with substances (i.e. proteins, lipids, polysaccharides, and synthetic or natural compounds) able to absorb or encapsulate, among the other, different botanicals a.i. (Gupta et al. 2023). The efficacy of these nanostructures against several pests was well investigated (Athanassiou et al. 2018). Polyethylene glycol nanoparticles (PEG-NPs) loaded with garlic EO showed high efficacy against *T. castaneum* and also exhibited a gradual release of the a.i. over time (Yang et al. 2009). Similarly, garlic EO lipid nanoparticles were tested against *Phthorimaea operculella* Zeller (Lepidoptera: Gelechiidae), showing a negative effect on adult longevity (Ibrahim et al. 2021). Heidary et al. (2022) developed *Thymus daenensis* EO nanocapsules and highlighted a high mortality rate against the cabbage aphid *Brevicoryne brassicae* L. (Hemiptera, Aphididae). The activity of *Zanthoxylum riedelianum* EO-based nano-encapsulates can also produce an ovideterrent effect against *Bemisia tabaci* Gennadius (Hemiptera: Aleyrodidae) (Pereira et al. 2018).

1.3.3 Fabrication methods

Botanical-based nanoformulations can be developed through two different approaches namely bottom-up and top-down (Fig. 2) (Donsì and Ferrari 2016; Modafferi et al. 2024b). Bottom-up

approaches (i.e. self-emulsion and phase inversion temperature or concentration) allow these nanoformulations to be obtained by combining molecules into complex structures due to the natural interaction among them. These approaches require low energy input and allow the development of formulations with unique properties. However, these approaches have some limitations such as the need for large amounts of co-formulants in order to obtain stable formulations and not all botanicals can be nano-formulated (Tadros et al. 2004; Sagalowicz and Leser 2010). On the other hand, top-down approaches (i.e., sonication, high-pressure homogenisation, or microfluidisation) due to high-energy systems allow reducing the size of raw formulations into fine and homogeneous particles. The advantages of employing these approaches lie in the possibility of using a low amount of co-formulants and most botanical a.i. can be nano-formulated. The ability to use these approaches in large-scale production could promote wider availability of these biopesticides in real operating conditions (Barradas and de Holanda e Silva 2020; Modafferi et al. 2024b).

Figure 2: Difference in terms of obtained particle size, energy, and amount of co-formulants between bottom-up and top-down approaches.



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CHAPTER 2

Aims of the thesis

Citrus production holds significant economic importance on a global scale, with over 12 million hectares of land dedicated to its cultivation. The industry not only supports international trade but also plays a vital role in boosting local production, providing livelihoods for millions of farmers and contributing to regional economies. However, several pests attack these plants and cause huge economic damage. Among these, *Planococcus citri* Risso, *Delottococcus aberiae* DeLotto (Hemipetera: Pseudoccoccidae), and *Ceratitis capitata* (Wiedemann) (Diptera: Tephritidae) are considered citrus key pests and their optimal management is mandatory. Although biological control strategies based on the use of natural enemies (such as predators or parasitoids) increased in recent years, the use of conventional insecticides is still the main control strategy. However, the indiscriminate use of these substances has led to several consequences on the environment, human health and adverse effects towards non-target organisms. For these reasons, in the last decade, several researchers have investigated the efficacy of alternative substances of natural sources against plant pests. Among these, the proven potential of essential oils (EOs) as natural insecticides, particularly in the context of nano-formulations, is enough to translate research into real-world applications. The general aim of the PhD Thesis was to develop and test new EO-based nanoformulations against different pests evaluating the adverse effects towards non-target organisms. *Allium sativum* EO was mainly used in the studies due to its demonstrated efficacy and stability in nano-emulsion formulations, as highlighted in Chapters 4-7. In addition, this EO showed higher insecticidal activity against citrus pests compared to the other EOs tested in preliminary trials. One of the primary aims was to develop high-performance nano-emulsions capable of incorporating a high concentration of EO as a.i. while maintaining long-term stability coupled with high EO:surfactant ratio. This involves optimizing emulsification techniques and conducting detailed assessments of the physical properties of the nanoformulations, such as particle size, polydispersity index (PDI), and surface charge. By ensuring the stability and efficacy of these formulations, this research aims to overcome one of the major limitations of EO-based insecticides, which is their tendency to degrade or lose potency over time and reduce phytotoxicity. The research also aims to investigate the potential of EO-based nanoformulations to activate plant defense mechanisms as well as the effect on detoxifying enzymes in *C. capitata*. Beyond their direct insecticidal effects, these formulations may induce systemic resistance in plants, enhancing their natural ability to defend against pests. This represents a novel approach to integrated pest management (IPM), combining direct pest control with the activation of plant immunity. Finally, the thesis evaluates the ecological safety of EO-based nanoformulations on non-target organisms,

such as pollinators and natural predators. Ensuring the safety of beneficial species is essential for sustainable pest management, and this research assesses the impact of the formulations on non-target organisms, including honeybees (*Apis mellifera*) and predatory insects like *Cryptolaemus montrouzieri*. Below, a general overview of the different research activities carried out during the PhD period was briefly summarised while the whole research is reported in chapters 3-7.

Chapter 3. Ecological costs of botanical nano-insecticides: a comprehensive overview about the use of botanicals as insecticides particularly those encapsulated inside nano-delivery systems (i.e. nanostructures and nano-emulsions) was drawn up. The manuscript provides an outline of the efficacy of these botanical-based nano-insecticides against target organisms. Furthermore, this paper underlined the main pros and cons related to the use of these substances under real operating conditions such as the impact on non-target organisms (i.e. pollinators, natural enemies, mammals, aquatic organisms, and invertebrates), and their sustainability and potential. This chapter enabled the establishment of optimised protocols for subsequent trials, while also facilitating the identification and screening of the most promising a.i. for use in the experiments. Additionally, this knowledge laid the groundwork for refining high-energy emulsification techniques and ensuring the long-term stability of nano-emulsions, thereby guiding the development of innovative and sustainable pest control strategies for subsequent phases of the research.

Chapter 4. High-energy emulsification of *Allium sativum* essential oil boosts insecticidal activity against *Planococcus citri* with no risk to honeybees: the best method to obtain a stable and effective *Allium sativum* EO-based nano-emulsion against citrus pests, while mitigating the adverse effects on non-target organisms were investigated. In detail, four *A. sativum* EO-based nano-emulsions were developed using different approaches (such as self-emulsification process, sonication, high pressure microfluidisation and a mixture of the three processes). The different formulations were subjected to physical characterisation over time to assess which one was the most stable in terms of size, PDI and surface charge. Among the different developed nanoformulations, the most effective one, i.e. *A. sativum* EO-based nano-emulsions was tested to estimate the lethal doses (LD_{50} and LD_{90}) against the target pest and to evaluate their safety to honeybees.

Chapter 5. Bioactivity of *Allium sativum* essential oil-based nano-emulsion against *Planococcus citri* and its predator *Cryptolaemus montrouzieri*: The aim of this chapter was to develop a stable *A. sativum* EO-based nano-emulsion with a high EO: surfactant ratio (3: 1) as eco-friendly pest management tool. The developed nano-emulsion was developed through the high-energy microfluidisation process using a low amount of surfactants, known for their phytotoxic effects. Dynamic light scattering (DLS) analysis was used to assess the physical properties of this nano-delivery system (i.e. size, PDI and surface charge). The EO-based formulation was then tested in two different bioassays (i.e. direct and indirect exposure) against the 2nd instar of *P. citri* to simulate real-world conditions. The estimated lethal doses (LD_{50} and LD_{90}) of *P. citri* were used to assess the undesired effects towards the coccinellid predator, *Cryptolaemus montrouzieri* Mulsant (Coleoptera: Coccinellidae).

Chapter 6. Green pest control strategies: Essential oil-based nano-emulsions for *Delottococcus aberiae* De Lotto (Hemiptera: Pseudococcidae) management: The aim of this chapter was to develop three different EO-based nano-emulsions investigating their efficacy against the invasive mealybug *D. aberiae*. In addition, the effects of the most effective nano-emulsions towards *C. montrouzieri* and on citrus plants were evaluated. Specifically, garlic (*Allium sativum*), clove (*Syzygium aromaticum*) and eucalyptus (*Eucalyptus camaldulensis*) EO-based nano-emulsions were developed and physically analysed using high-energy (i.e. microfluidisation) DLS analysis respectively. The formulations were subjected to a preliminary screening to evaluate their toxicity against the target pest and then to estimate the lethal doses of the most effective EO-based nano-emulsion (i.e. garlic one). The estimated LD_{50} and LD_{90} were used to evaluate the toxic effects in residual contact toxicity trials against *C. montrouzieri* adults and in phytotoxicity bioassays on *Carrizo citrange* (*C.* ×*aurantium* ×*C. trifoliata*) plants.

Chapter 7. Metabolic and microbial responses of *Ceratitis capitata* to essential oil-based nanoemulsions: implications for pest management: This work aimed at the evaluation of EO-based nano-emulsions against the Mediterranean fruit fly *Ceratitis capitata* (Wiedemann) (Diptera: Tephritidae). The EO-based nano-emulsions were obtained through a top-down approach (i.e. microfluidisation technique) and physically characterised by DLS analysis. The nano-emulsions were used in the preliminary trials to evaluate the biological activity against the target pest and to estimate the lethal doses (LD_{50} and LD_{90}). The LDs were then used to evaluate the effects on the

transcription levels of several genes involved in insecticide resistance and on the gut microbiota, highlighting a dual effect (metabolic stress-microbiota disruption).

CHAPTER 3

Ecological costs of botanical nano-insecticides

(This is the published version in

Current Opinion in Environmental Science & Health

https://doi.org/10.1016/j.coesh.2024.100579)

Available online at www.sciencedirect.com

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Current Opinion in
Environmental Science & Health

Ecological costs of botanical nano-insecticides

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Botanical nano-insecticides are a trend in pest control. The natural origin of the active substances, alongside with the methodological approach granted by nanotechnologies are a promising combination of innovation and eco-sustainability, hot topics in the context of ecological transition in agriculture. Nevertheless, their field application is still limited, due to production challenges and risk assessment concerns. Nano-formulations have some advantages over traditional bioinsecticides, including increased bioactivity and persistence, and slow-release rates. Recent research reported promising insecticidal activity of nano-emulsions, micro-emulsions, and nanoparticles loaded with different botanical extracts, oils, and essential oils. Though, despite their proven efficacy against insect pests and vectors, a limited number of studies investigated their safety towards nontarget organisms and fate in the environment. This mini-review provides an overview of the side-effects of botanical nano-insecticides and the main challenges to improve their sustainability in term of ecological and production cost.

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Current Opinion in Environmental Science & Health 2024, **42:**100579

This review comes from a themed issue on **Environmental Toxicology 2025: Non-target effects of Bio-insecticides**

Edited by **Raul Narciso C. Guedes**, **Giovanni Benelli**, **Nicolas Desneux** and **Evgenios Agathokleous**

For a complete overview see the Issue and the Editorial

https://doi.org/10.1016/j.coesh.2024.100579

Introduction

In the last decades, ecofriendly pest control has become a key topic to safeguard crop production and ensure food security worldwide. The adverse consequences of the extensive use of synthetic insecticides, naming pesticide resistance, water and soil contamination, and the negative effects on human health and nontarget species, prompted stakeholders to explore innovative and more selective eco-friendly tools [1]. Combining nanotechnological approaches with botanical active substances is a leading trajectory for developing and commercializing innovative bioinsecticides [2]. Synthetic insecticides are usually preferred by farmers because of their superior effectiveness to control pests compared to botanical insecticides [3]. Nevertheless, nano-bioinsecticides often showed higher insecticidal activity, biodegradability, and controlled or targeted release, if compared to the synthetic counterparts [3].

Despite many studies on the efficacy of botanical extracts as insecticides, only a few commercial products are available because some disadvantages limit their use in field conditions [4]. Most bioactive botanicals are secondary metabolites synthesized by plants as a mixture of different molecules, called phytocomplex. Their natural origin secures biodegradability, although it also determines rapid degradation and low persistence, sometimes associated with flammability, poor solubility in water, and phytotoxicity [3,5].

Those negative characteristics are some of the challenges to overcome for the ecological transition in agriculture, which could be mitigated through the application of nanotechnology [6]. Granting improved persistence and efficacy on target pests, botanical nano-insecticides might also prove increased bioactivity toward nontarget organisms, as well as environmental concerns. Nevertheless, those aspects are usually neglected; besides, the selectivity of botanicals is commonly acknowledged, occasionally without a solid scientific ground [7].

In this scenario, the present mini-review explores the use of botanical nano-delivery systems in managing insect pests and vectors focusing on the associated ecological challenges. The primary objective is to define these systems and emphasize their environmental impact, including toxicity to nontarget organisms and

Given the role as Guest Editor, Giovanni Benelli had no involvement in the peer review of the article and has no access to information regarding its peer-review. Full responsibility for the editorial process of this article was delegated to Nicolas Desneux.

long-term ecological effects. Nano-delivery systems, such as nanoparticles, nano-emulsions, and nano-capsules are described, and their respective characteristics are highlighted. The recent literature on side-effects of nanoformulations containing active substances obtained from plants or industrial botanical byproducts, rather than chemically-synthesized single molecules (e.g. terpenes) originally identified from botanical sources, is reviewed. Nanodelivery systems where botanicals were used as co-formulants or carriers were disregarded. Lastly, the sustainability of manufacturing processes leading to the production of botanical nano-systems and the future challenges for their development and commercialization are highlighted.

Botanical nanoinsecticides

Overall, nanotechnology refers to materials with dimensions ranging in the nanometric scale (between 1 and 100 nm). Instead, approaches for nanoinsecticidal design refer to nanodelivery systems with particle sizes lower than 1000 nm [8]. Nano-formulations applied to the development of botanical nanoinsecticides consist of two main groups: (i) nano-emulsions and micro-emulsions, and (ii) nanoparticles. Due to their ease of preparation and industrial scalability, both groups can work for innovative pest control formulations [9]. Nanodelivery systems offer several advantages over traditional botanical pesticides, improving surface coverage, dispersibility, controlled release kinetics, and enhancing penetration through the insect cuticles and the plant tissues. Overall, those features can reduce the amount of the active substance required in field conditions, meanwhile increasing pest control efficiency in crop protection [6]. Therefore, the efficacy of botanical nano-insecticides was well studied against different crop and stored product pests, as well as insect vectors [10,11].

Nano and microemulsions

Among all the nanodelivery systems, nanoemulsions (NEs) and microemulsions (MEs) have been the most common in the design of nano-insecticides from botanical extracts. NEs and MEs are dispersed systems composed of a mixture of immiscible liquids (e.g. oil and water) stabilized by an emulsifying agent. NEs and MEs can be mainly developed through bottom-up and top-down approaches [12]. Top-down methods, utilizing high-energy systems (i.e. sonication, high-pressure homogenization, or micro-fluidization) offer some advantages, such as precise control over physical properties and the scalability for large-scale production. Bottom-up approaches require low-energy (e.g. self-emulsification, phase inversion concentration or temperature, precipitation) and are advantageous in terms of versatility and the ability to develop materials with unique properties. The choice of the adequate method is a key issue to balance NE and ME characteristics and production's energy costs [13].

NEs and MEs can be applied to insecticidal formulations to make some lipophile botanical extracts miscible with water. This is the case of several vegetable oils, such as neem and castor oil, and essential oils (EOs). Some of those botanical extracts are quite common pests and vector control tools, although some drawbacks can impair their efficacy in field conditions [14].

Nanoparticles

Nanoparticles (NPs) refer to solid or liquid nanomaterials, such as nanospheres and nanocapsules, derived from substances capable of absorbing or encapsulating active substances. Various approaches can be used to develop NPs, including precipitation, solvent evaporation, and melt dispersion, utilizing materials like poly-ε-caprolactone (PCL), polyethylene glycol (PEG), silica, chitosan, and zein [15]. These nanoformulations can absorb, dissolve, encapsulate or entrap different botanical active substances, such as EOs, pyrethrins, rotenone, neem oil, and other plant extracts. Their insecticidal efficacy was proven against several crop and stored product pests, as well as on mosquito vectors [16]. The materials used to produce the nanocapsules or nanospheres can alter several physicochemical characteristics of the botanical extracts, influencing the release rate, the persistence and the bioavailability of the phytocompounds. However, the inclusion of active substances inside a protective shell can also assume undesired effects, like bioactivity reduction and residual presence on crops.

Are botanical nanoinsecticides ecofriendly and sustainable?

The impact of botanical nanoinsecticides for pest control on the biota of both natural and agroecosystems is still far to be clarified. However, nanoformulated substances could potentially impact several ecological components and processes. Recently, the environmental impact and fate of nanopesticides have been reviewed, dealing with different aspects of eco-sustainability [17–19]. Nevertheless, those studies mainly focused on metallic NPs or nanoformulations containing synthetic insecticides, highlighting the need of a focused revision of the literature on botanical nanoformulations. However, some of the considerations and concerns defined for synthetic and metallic nanomaterials cannot be extended to plant-based nano-insecticides since, for their nature, those are more prone to biodegradation.

Side effects on nontarget species

The potential adverse effects of botanical-based nano-insecticides towards nontarget organisms, including arthropods (such as natural enemies and pollinators), aquatic and soil organisms and microorganisms, plants,

as well as humans, are still uncertain. Indeed, the undesirable effects of plant-based nanopesticides depend on several factors, including the particle number, concentration, size, distribution, and application rate [20].

However, the selectivity of bioinsecticides is commonly acknowledged and, thus, extended to nanobiopesticides. Nevertheless, current literature about the nontarget impact of botanical insecticides reported contrasting results [7], and the same may also be supposed for their nanoformulations. For example, pyrethrum-based nanopesticides did not significantly affect honeybee survival, whereas unformulated pyrethrum reduced bee longevity and caused morphological alterations in the midgut [21]. Natural enemies, such as insect predators and parasitoids, merit attention when considering the use of botanical nanoinsecticides for crop protection [22–24]. Among invertebrates, aquatic microcrustaceans, like *Daphnia magna* Straus, and earthworms, such as *Eisenia fetida* Savigny, are recognized model organisms for ecotoxicological study, which can help to understand the activity of xenobiotics on different organisms of the agroecosystem trophic chain [25–27]. Furthermore, botanicals may influence soil microorganisms, including soil nitrogen cycle microbiota [28], while their nanoformulation could be more selective [29]. Lastly, the safety of several EO-based NEs toward mammalian [30–32] and human cell lines has been reported [31–34]. The impact of botanical nanoinsecticides toward nontarget organisms is detailed in Table 1. To date, severe acute toxicity has not been reported for tested nanoinsecticides, although further research is required to safely apply botanical nanosubstances in the field.

Environmental persistence

A further key issue about botanical nanoinsecticides is the environmental impact assessment, in particular their accumulation in soil, water, plants, and foods. In this context, the most common methodologies for studying the behavior and fate of nanoformulated pesticides in soil and water were discussed [48]. In aquatic and soil environments, the physicochemical properties of the botanical nanomaterials can alter some physicochemical characteristics and influence both the biotope and the biocenosis [45,49]. The European Food Safety Authority (EFSA) published a guideline on the food safety assessment of nanoformulations applied in agriculture [50]. Since the nanoinsecticides may not be environmentally safe, deeper knowledge about the fate of botanical nanosystems is required. Life cycle assessment (LCA) in soil, water, and plants could be an interesting approach to estimate the safety of botanical nanomaterials, since in open fields their fates depend on their physicochemical properties (e.g. particle size, surface chemistry, and charge) and bioactivity, alongside with field conditions (e.g. soil/water composition and climate), which can alter biodegradation and bioavailability processes [51], including soil enzymatic activity [24,52].

The possible bioaccumulation in the environment and the biomagnification through the trophic chain are the most concerning aspects related to the field application of botanical nanoinsecticides, although few studies have tried to investigate those aspects [53,54]. Nanoformulations could increase the soil half-life of some synthetic pesticides up to 2-fold, with recorded bioaccumulation in earthworms and plants [55]. On this basis, a similar persistence trend cannot be excluded also for botanical nanoformulations, which can stay active in organic substrates, such as soil and water, for quite a long period due to their controlled release rate and increased stability. In our opinion, long-term studies to fully understand the environmental persistence and potential bioaccumulation of botanical nanoinsecticides is a crucial issue deserving further research.

Sustainability and commercial challenges

Among the characteristic limitations of plant-based nanopesticides, the quantity of coformulants used to stabilize the nanoformulations is a key issue. Some of these substances are known to adversely affect plant growth and cell membrane permeability at high concentrations [56]. The use of natural *versus* synthetic emulsifiers and coformulants should be preferred to improve the complete biodegradation of the nanoformulation in open field conditions [57] (Table 2).

On the other hand, while reducing the use of synthetic coformulants, the botanical active substance included in the nanopesticidal formulation should be highly concentrated; otherwise, high volumes of nanoformulants would be needed for real-world use, causing issues during storage, transport, and application. There are only a few stable nanoinsecticides formulated with high ratios of botanical active substances (i.e. >15%) [e.g. 22,23], although this aspect needs to be further improved to match commercial requirements.

Lastly, the processes employed to produce botanical nanoformulations merit attention. Besides the promising physicochemical characteristics of nanoformulates, some of the proposed approaches require expensive external inputs, such as high energy costs, as well as expensive materials [57]. The environmental impact of the industrial production of nanoinsecticides involves land use for raw material production, carbon and water footprint, as well as waste management. Indeed, when accounting for the sustainability of those insecticides, all these aspects (i.e. from field to commercialization) should be considered. In this framework, the extraction methods of the botanical active substances, the

Table 1

Side effects of botanical nanoinsecticides on nontarget organisms.

Plant species (Family)	Active substance	Type of formulation	Target species	Major results	Nontarget species	Main effects	Reference
Schinus terebinthifolius (Anacardiaceae)	EO	NE	*Culex pipiens*	Larvae: LC_{50} = 6.8 μl/L, LC_{95} = 13.2 μl/L Adults: LC_{50} = 5.3 μl/L, LC_{95} = 11.3 μl/L Repellent at all tested doses (μg/cm^2).	*Gambusia affinis* *Eisenia fetida*	LC_{50} = 3042.7 μl/ml LC_{95} = 5614.7 μl/ml Not detected effects on mortality	[35]
Allium sativum (Amaryllidaceae)	EO	NE	*Planococcus citri*	24h: LC_{50} = 0.76%; LC_{90} = 1.378% 48h: LC_{50} = 0.65%; LC_{90} = 1.1%	*Apis mellifera*	Survival: 100%	[13]
				Direct: LC_{50} 0.248%, LC_{90} = 0.967% Residual: LC_{50} = 0.782%, LC_{90} = 1.088%	*Cryptolaemus montrouzieri*	Survival = 90% ± 5.37 at LC_{90}; 84.44% ± 6.7 at 1.25%	[22]
			Tuta absoluta	Eggs: LC_{50} = 0.124%, LC_{90} = 0.772% Larvae: 100% mortality at 3%, 7.78 mortality at LC_{50} eggs Repellent	*Nesidiocoris tenuis* Tomato plants	Mortality: undetected Reduced progeny in treated plants Undetected	[23]
Acmella oleracea (Asteraceae)	EO	NE	*Culex quinquefasciatus*	LC_{50} = 407.5 μL/L	Mammalian fibroblasts and microglia cells	Low level of cytotoxicity and anti inflammatory effect	[33]
Ageratina adenophora (Asteraceae)	4,7-dimethyl-1-(propan-2-ilidene)–1,4,4a,8a-tetrahydronaphthalene-2,6(1 H,7 H)-dione (DTD)	NE	*Spodoptera frugiperda*	*S. frugiperda*: 72h LC_{50} = 47.02 mg/L, 96h LC_{50} = 24.02 mg/L	Cell	Low toxicity	[36]
			Spodoptera litura	*S. litura*: 72h LC_{50} = 14.03 mg/L, 96h LC_{50} = 0.79 mg/L	Earthworms	7d LC_{50} = 40.46(mg/kg); 14d LC_{50} = 37.57 (mg/kg)	
			Ostrinia furnacalis	*O. furnacalis:* 72h LC_{50} = 33.89 mg/L, 96h LC_{50} = 2.19 mg/L	*Zea mais*	Undetected	
Carlina acaulis (Asteraceae)	EO & carlina oxide	NE	*Culex quinquefasciatus*	LC_{50} = 579.1 μL L^{-1}; LC_{90} = 791.3 μL L^{-1}	Human cells	Low toxicity	[31]
				Sublethal effects: LC_{16} (384.5 μL L^{-1}) = 100% mortality after 18 days	Wistar rats	Undetected toxicity (LC_{50} = 5000 mg/kg)	
Tanacetum cinerariifolium (Asteraceae)	Pyrethrins (commercial product)	solid lipid NP	-	–	*Apis mellifera*	Undetected on longevity and digestive cells	[21]
					Lithobates catesbeianus	Genotoxic	[37]
	Pyrethrins (commercial product)	ME	*Aphis gossypii*	Population reduction (%): 3.1 g a.i/hl after7d (90.68%); 1.86 g a.i/hl after 7d (77.66%)	*Coccinella septempunctata*	Undetected	[38]
					Macrolophus pygmaeus	Undetected	

Pimpinella anisum	EO	ME	*Culex quinquefasciatus*	LC_{50} = ranging from 1.45 to 4.01 ml/L	*Daphnia magna*	Low toxicity	[39]
Trachyspermum ammi				LC_{90} = ranging from 1.81 to 6.48 ml/L	*Tubifex tubifex*	High toxicity	
Crithmum maritimum (Apiaceae)					*Eisenia fetida*	Undetected	
Smyrnium olusatrum (Apiaceae)	Isofuranodiene	ME	*Culex quinquefasciatus*	24h: LC_{50} = 17.7 ml/L, LC_{90} = 39.1 ml/L	*Daphnia magna*	Mortality: 18.7% (32 ml/L)	[40]
				7d: LC_{50} = 4.1 ml/L, LC_{90} = 11.3 ml/L	*Eisenia fetida*	Undetected mortality	
Cannabis sativa (Cannabaceae)	EO	NE	*Culex quinquefasciatus*	LC_{50} = 72.2 ppm; LC_{90} = 207.2 ppm	*Daphnia magna*	Mortality <16% at LC_{90}	[41]
Cupressus sempervirens (Cupressaceae)	EO	NE	*Culex quinquefasciatus*	Larvae: LC_{50} = 11.4 μg/ml, LC90 = 19.7 μg/ml	*Gambusia affinis*	LC_{50} = 1488.4 μg/ml	[42]
				Adults: LC_{50} = 7.2 μg/l, LC_{90} = 13.1 μg/l; Repellent at all tested doses (μg/cm^2).		LC_{90} = 2425.5 μg/ml	
Croton linearis (Euphorbiaceae)	EO	NE	*Aedes aegypti*	LC_{50} = 17.86 μg/mL	Human cells	Undetected effects (LC_{50} > 2000 mg/kg)	[32]
Aeollanthus suaveolens (Lamiaceae)	EO	NE	*Aedes aegypti*	24h: LC_{50} = 54.23 μg/mL, LC_{90} = 96.96 μg/mL	*Mus musculus*	Undetected effects (LC_{50} > 2000 mg/kg)	[30]
				48h: LC_{50} = 46.06 μg/mL, LC_{90} = 75.31 μg/mL			
Mentha piperita (Lamiaceae)	EO	Polymeric NP	*Sitophilus oryzae*	*S. oryzae:* LC_{50} = 130.5 μg/cm^2, LC_{90} = 327.61 μg/cm^2	*Artemia salina*	LC_{50} = 24.74 ppm, LC_{90} = 47.72 ppm	[43]
			Lasioderma serricorne	*L. serricorne:* LC_{50} = 162.04 μg/cm^2, LC_{90} = 348.86 μg/cm^2			
			Culex pipiens	*C. pipiens:* LC_{50} = 66.02 μg/cm^2, LC_{90} = 122.43 μg/cm^2			
Mentha spicata (Lamiaceae)	EO	Chitosan NP	*Callosobruchus maculatus*	*C. maculatus:* LC_{50} 56 μL/L	Vero cell line	Undetected effects	[34]
			Sitophilus granarius	*S. granaries:* LC_{50} 47 μL/L			
Persea venosa (Lauraceae)	EO	NE	*Dysdercus peruvianus*	LC_{50} = 28.73 μg/μL	*Apis mellifera*	Undetected mortality	[44]
					Partamona helleri	Undetected mortality	
Azadirachta indica (Meliaceae)	Neem oil	zein NP	-	–	*Allium cepa*	Decreased mitotic index	[28]
						Slightly increased damage index	
					Soil nitrogen cycle microbiota	Undetected	
					Caenorhabditis elegans	Undetected	
		PLC NP	-	–	Soil microbiota	Undetected until 300 days	[29]
					Zea mays	Dose-responsive phytotoxicity	
					Allium cepa		
	Neem gum	Nano-suspension	*Helicoverpa armigera*	LC_{50} = 10.20 ppm; LC_{90} = 32.68 ppm	*Eudrilus eugeniae*	Undetected mortality	[26]

(*continued on next page*)

Table 1. (*continued*)

				Antifeedant Pupal toxicity			
			Spodoptera litura	LC_{50} = 12.49 ppm; LC_{90} = 36.68 ppm; Antifeedant Pupal toxicity			
Myristica fragrans (Myristicaceae)	EO	chitosan NP	-	–	Rice	Reduced peroxidase activity	[45]
						Undetected phytotoxicity on seed germination	
					Mice	LC_{50} = 9231.89 μL/kg	
Syzygium aromaticum (Myristicaceae)	EO & Eugenol	zein NP	*Drosophila melanogaster*	Mortality after 14 days: 100% (Zn-EO) & >60% (Zn−Eu)	*Caenorhabditis elegans*	Low toxicity	[46]
Cymbopogon martinii (Poaceae)	EO	Polymeric NP	*Sitophilus oryzae*	*S. oryzae:* LC_{50} = 128.82 μg/cm^2, LC_{90} = 209.37 μg/cm^2	*Artemia salina*	LC_{50} = 30.74 ppm, LC_{90} = 69.97 ppm	[43]
			Lasioderma serricorne	*L. serricorne:* LC_{50} = 141.08 μg/cm^2, LC_{90} = 321.81 μg/cm^2			
			Culex pipiens	*C. pipiens:* LC_{50} = 53.12 μg/cm^2, LC_{90} = 105.55 μg/cm^2			
Citrus sinensis	EO	NE	-	–	*Nesidiocoris tenuis*	*C. reticula* causes lethal and sublethal effects	[24]
Citrus reticulata					Soil activity	Undetected	
					Tomato plant	Undetected	
		PEG NP			*Nesidiocoris tenuis*	*C. reticula* causes lethal and sublethal effects	
Citrus limon (Rutaceae)					Soil activity	Undetected	
					Tomato plant	Undetected	
Murraya koenigii (Rutaceae)	EO	NE	*Aedes aegypti*	LC_{50} = 11,8 μg/ml, LC_{90} = 22,6 μg/ml	*Allium cepa*	Antiproliferative	[47]
Siparuna guianensis (Siparunaceae)	EO	Chitosan NP	*Aedes aegypti*	Mortality 7d: 100%	*Poecilia reticulata*	Mortality 24h: <30% (0.83 mg/mL)	[27]
					Danio rerio	Mortality 24h: <30% (0.45 mg/mL)	

Table 2

Common coformulants used to produce nanoinsecticides.

Substance	Description	Advantages	Disadvantages
Polysorbates (Tween)	Ethoxylated sorbitan esterified with fatty acids Amphiphilic, synthetic nonionic surfactants	Biodegradable in soil Used as wetter on agricultural crops	Phytotoxicity (high concentrations) High amount required Expensive
Sorbitan esters (Span)	Sorbitan esterified with fatty acids Amphiphilic, synthetic nonionic surfactants	Biodegradable in soil	Phytotoxicity (high concentrations) High amount required Expensive
Polyethylene glycol (PEG)	Linear polyether Synthetic coating	Biocompatible Soluble in water and most organic solvents	Dry formulations Moisture degradation
Polycaprolactone (PCL)	Linear aliphatic polyester Synthetic adsorbent, coating	Biocompatible Long term persistence	Hydrophobic Residual problems
Polyvinyl alcohols	Vinyl polymer Synthetic thinner, emulsifier	Biocompatible Water-soluble	Low persistence High degradability
Silica	Amorphous mineral, natural or synthetic Adsorbent	Biostimulant on several crops Natural or synthetic	High temperature ROS generation Limited water solubility
Alginates	Linear anionic polysaccharide Adsorbent, coating, emulsifier	Natural product Water soluble	Intrinsically variable structure Not degradable by mammals
Chitosan	Linear polysaccharide Emulsifier, coating	Natural product Biostimulant on several crops	Insoluble in water and organic solvents Variable molecular weight and deacetylation degree
Zein	Plant protein isolated Adsorbent, coating, emulsifier	Natural product Soluble in organic solution	Expensive Low stability, enzymatic degradation
Pectin	Linear polysaccharide Emulsifier, coating	Natural product Soluble in water	Intrinsically variable structure Strong retention

production technologies, byproduct disposal, but also the storage requirements (i.e. refrigeration), could greatly impact on the sustainability of botanical nano-insecticides [5,58]. Researchers are beginning to face these challenges, as for the bioproduct management, proposing alternative and more sustainable methods (i.e. solvent-free extraction) [59].

Conclusions

Although the potential of botanical biopesticides has been recognized by a growing body of literature, to date only a few biopesticides are commercialized and used by stakeholders [4]. This could be explained by the limited effectiveness of botanical insecticides in the field. Nevertheless, the dangers associated with synthetic insecticides became clear once residuals accumulated in soil, water, and plants and contaminated the environment, highlighting that in several cases the risks have outweighed their benefits [58].

A similar misconnection between research and market is apparently occurring also for botanical nano-insecticides. Undoubtfully, their widespread adoption and commercial development hinge on addressing ecological concerns, optimizing formulation processes, and ensuring compatibility with environmental and human health standards. Therefore, risk assessment of nanomaterials has become a trend challenge to face in the next future. However, common methodologies and frameworks for risk assessment of botanical nanoinsecticides are still lacking, posing difficulties to assess and compare their side effects on nontarget species, as well as their impact and risks on aquatic and terrestrial ecosystems, biodiversity, and food webs.

Overall, besides the encouraging results available from the literature on their ecotoxicology, the risk assessment and the sustainability of botanical nanoinsecticides need further investigation to validate both industrial scalability and environmental safety.

Author's contributions

Antonino Modafferi Visualization, Writing- Original draft preparation. **Giulia Giunti** Conceptualization, Visualization, Writing- Original draft preparation. **Giovanni Benelli** Validation, Writing- Reviewing and Editing. **Orlando Campolo** Conceptualization, Writing-Reviewing and Editing.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Giovanni Benelli is a Guest Editor of the Special Issue "*Environmental Toxicology 2025: Non-target effects of Bio-insecticides*". Giovanni Benelli has not been involved in decisions about the present article, peer review of the present submission has been handled independently of the guest editor and his research group. The other authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this article.

Data availability

No data were used for the research described in the article.

Acknowledgements

This study was funded by European Union – Next Generation EU within the project PRIN 2022 - Managing *Varroa destructor* through selective acaricides (DD n. 104 del 02-02-2022 PRIN 2022 - 2022XPRCMS - M4. C2.1.1. - CUP Master I53D23004600006). This manuscript reflects only the authors' views and opinions, neither the European Union nor the European Commission can be considered responsible for them.

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CHAPTER 4

High-energy emulsification of *Allium sativum* essential oil boosts insecticidal activity against *Planococcus citri* with no risk to honeybees

(This is the published version in

Journal of Pest Science

https://doi.org/10.1007/s10340-024-01800-2)

Journal of Pest Science
https://doi.org/10.1007/s10340-024-01800-2

ORIGINAL PAPER

High-energy emulsification of *Allium sativum* essential oil boosts insecticidal activity against *Planococcus citri* with no risk to honeybees

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Received: 9 February 2024 / Revised: 31 May 2024 / Accepted: 5 June 2024

Abstract

The ecotoxicological consequences of synthetic pesticides have encouraged stakeholders to search for eco-friendly pest control tools, like essential oils (EOs). Nano-delivery systems (nanoparticles and nano-emulsions) seem ideal for developing EO-based biopesticides, although production processes should be standardized and implemented. In this study, nano-emulsions loaded with a high amount of *Allium sativum* L. EO (15%) were developed using different mixed bottom-up/top-down processes. Garlic EO was chemically analyzed by gas chromatography-mass spectrometry (GC-MS) and formulations were physically characterized using Dynamic Light Scattering (DLS) apparatus. The insecticidal activity against *Planococcus citri* Risso (Hemiptera: Pseudococcidae) and selectivity toward *Apis mellifera* L. (Hymenoptera: Apidae) worker bees was evaluated. Garlic EO was mainly composed of sulphur components (96.3%), with diallyl disulphide and diallyl trisulphide as the most abundant compounds (37.26% and 28.15%, respectively). Top-down processes could produce stable nano-emulsions with droplet size in the nanometric range (< 200nm) and good polydispersity index (PDI < 0.2). In contrast, the bottom-up emulsion was unstable, and its droplet size was around 500nm after 24 hours. High-energy emulsification processes significantly increased the residual toxicity of garlic EO against 3rd instar *P. citri* nymphs, whereas the developed formulations were harmless to *A. mellifera* workers in topical application. This study confirmed that the production process significantly affected the physical properties and efficacy against target pests. The lack of adverse impact on honeybees denotated the potential of these formulations as bioinsecticides in organic and/or IPM programs, although further extended ecotoxicological studies are necessary.

Keywords Botanicals · Bioinsecticides · Citrus mealybug · Ecotoxicology · Garlic essential oil · Nanotechnology

Introduction

In recent years, alternatives to synthetic pesticides for crop protection have been investigated due to environmental and health problems caused by the widespread use of these substances (Nenaah et al. 2015; Rizzo et al. 2020). Among the alternative solutions, botanical extracts as essential oils (EOs) could be the ideal candidates for the development of new eco-friendly bioinsecticides (Franzios et al. 1997; Priestley et al. 2003; Raybaudi-Massilia et al. 2006; Zhou et al. 2008; Campolo et al. 2020; Ben Abdallah et al 2023).

Despite the efficacy of different EOs under laboratory conditions, some characteristics of these natural substances limited their use in real-field situations. Volatility, flammability, rapid degradation, poor solubility in water, and phytotoxicity are the challenges that should be overcome before

Communicated by Azucena Gonzalez-Coloma.

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Published online: 17 June 2024

using these substances as biopesticides (Pavela and Benelli 2016; Karalija et al. 2020).

These limitations of EOs can be mitigated through the development of more stable and effective formulations using nanotechnological interventions (Giunti et al. 2023). Among the different nano-formulations, EO-based nano-emulsions seem to be the ideal due to their easy preparation and scalability at the industrial level (Campolo et al. 2017).

Nano-emulsions are produced using bottom-up and top-down approaches (Donsì and Ferrari 2016). Bottom-up processes (e.g., self-emulsification, phase inversion composition or temperature, solvent demixing, etc.) are highly effective in combining very small molecules into more structured systems and producing nano-emulsions. Bottom-up approaches in general require a large amount of surfactant or co-surfactants to develop EO-based nano-emulsions at the nanoscale (droplet size < 200 nm) (Tadros et al. 2004; Sagalowicz and Leser 2010; Sugumar et al. 2014). Top-down approaches like sonication, high-pressure homogenization, high-pressure microfluidization, etc.) on the other hand, use high energy or pressure to create homogeneous nano-emulsions from large structured materials (Verma et al. 2009; Chan and Kwok 2011; Arole and Munde 2014). Top-down methods are generally considered more advantageous than bottom-up methods. They are easier to apply at industrial scale and reduce the occurrence of undesired effects, such as coalescence and flocculation, with reduced amount of surfactants and without co-surfactants and thickeners (Anton et al. 2008; McClements and Rao 2011; Santana et al. 2013; Barradas and de Holanda e Silva 2020).

Planococcus citri Risso (Hemiptera: Pseudococcidae), commonly known as citrus mealybug, is a polyphagous insect-pest that can affect agricultural and ornamental plants (Franco et al. 2004). This is one of the most important citrus pests due to its direct and indirect damages. The mealybug induces falling and deformation reduces plant growth and fruit size. The production of abundant honeydew that soils the fruits and attracts other undesirable insects, such as ants, can have significant economic consequences (Afifi et al. 2010; Zappalà 2010). Its management can be done through different strategies, such as agronomic practices and biological control using predators and/or parasitoids, but the primary control strategy is based on chemical insecticides (Ghaffari et al. 2017; Mansour et al. 2018).

The efficacy of EOs as insecticides has been investigated against several pests including *P. citri* (Koul et al. 2008; Mossa 2016; Campolo et al. 2018). Cloyd et al. (2009) highlighted that the EOs of cottonseed, cinnamon, rosemary, and lavender provided > 90% mortality of citrus mealybug. Similarly, EOs of *Pimpinella anisum* L., *Rosmarinus officinalis* L., *Mentha piperita* L., *Origanum onites* L., *Thymus vulgaris* L., and *Thymus capitatus* L. exhibited good fumigation efficacy against *P. citri* adults (Erdemir and Erler 2017, 2018; Alloui-Griza et al. 2022). Attia et al (2022) showed that the topical application of *Mentha pulegium* L. EO at 40.96 mg/L provided 100% mortality of *P. citri* nymphs.

In literature, the efficacy of *Allium sativum* (L.) EO has been extensively investigated against several pests. Garlic EO was highly effective against different mites such as *Tetranychus urticae* Koch, *Tetranychus truncates* Ehara (Acari: Tetranychidae), *Aceria oleae* (Nalepa), and *Tegolophus hassani* (Keifer) (Acari: Eriophyidae) (Attia et al. 2012; Mossa et al. 2018; Sararit and Auamcharoen 2020). Furthermore, *A. sativum* EO can also be used as a pesticide for nematode management (Catani et al. 2023). Its fumigant or contact toxicity was indeed confirmed against *Meloidogyne incognita* (Kofoid & White), *Meloidogyne javanica* (Treub) Chitwood, (Nematoda: Meloidogynidae), *Bursaphelenchus xylophilus* (Bursxy) (Nematoda: Aphelenchoididae) and *Panagrolaimus* sp. (Nematoda: Panagrolaimidae) (Park et al. 2005; Jardim et al. 2020; Oro et al. 2020; Galisteo et al. 2022, Nguyen et al. 2022). Among stored product pests, garlic EO and its major compounds revealed fumigant and contact toxicity against *Tribolium confusum* J. du Val and *Tribolium castaneum* (Herbest) (Coleoptera: Tenebrionidae), *Callosobruchus maculatus* (Fabricius) (Coleoptera: Bruchidae), *Sitophilus zeamais* Motschulsky and *Sitophilus oryzae* L. (Coleoptera: Curculionidae) (Huang et al. 2000; Yang et al. 2010; Douiri et al 2013; Chude and Chude 2020; Palermo et al. 2021). Crude garlic EO was also tested against crop pests although with contrasting results; as an example, it exhibited low toxicity against *Spodoptera littoralis* (Boisd.) (Lepidoptera: Noctuidae) larvae (Hamada et al. 2018). In contrast, Ricupero et al. (2022) produced a garlic EO-based nano-emulsion which highlighted good efficacy in terms of larval and egg mortality as well as oviposition deterrence against *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae). On this basis, the impact of formulation can be a critical point to investigate the bioactivity of this EO against major pests.

Among the different EOs used for the control of *P. citri*, garlic EO seems a good choice for the development of new control tools since this phytocomplex has been proved to exert good insecticidal activity without affecting the survival of its main predator *Cryptolaemus montrouzieri* Mulsant (Coleoptera: Coccinellidae) (Modafferi et al. 2024). In this study, the authors investigated the insecticidal activity of a garlic EO nano-emulsion produced with a single low energy method and its stability over time was determined (Modafferi et al. 2024). In view of the promising insecticidal activity of garlic EO, this study was aimed at developing various garlic EO-based emulsions comparing single or mixed bottom-up/top-down approaches, and maintaining the same high EO/surfactant ratio (3:1). The EO and the formulations were chemically and physically characterized using gas chromatography-mass spectrometry (GC–MS) and

dynamic light scattering (DLS) apparatus, respectively. The suitability of the different methods to produce stable nano-emulsion formulations was assesses in terms of their efficacy against 3rd instars of *P. citri* and selectivity toward adults of *A. mellifera* bees.

Materials and methods

Biological materials

Planococcus citri laboratory rearing was established from hundreds of specimens collected from citrus groves located in the Reggio Calabria province (Italy) in 2023. The mealybug colony was reared for several generations at the Entomology laboratory of the Department of Agriculture, University *Mediterranea* of Reggio Calabria. Insects were reared on organic butternut pumpkin fruit inside a climatic chamber under constant climatic conditions: 28 ± 1 °C, 70 ± 5% R.H., with a photoperiod of 12 h:12 h (L:D). Newly emerged (< 48 h) *Apis mellifera ligustica* Spinola (Hymenoptera: Apidae) worker bees used in the toxicity trial were collected from the experimental apiary of the Department of Agriculture, University *Mediterranea* of Reggio Calabria, Reggio Calabria, Italy. During the past three months, the apiary did not undergo any chemical treatment for *Varroa destructor* Anderson and Trueman (Acari: Varroidae) control.

Chemical characterization of *Allium sativum* EO

Commercial *Allium sativum* EO (GEO) was purchased from Esperis S.p.A. (Milan, Italy). The chemical characterization of the garlic EO was carried out following the methods described by Giunti et al. (2019). Briefly, a Thermo Fisher TRACE 1300 GC with a MEGA-5 capillary column (30 m × 0.25 mm; coating thickness = 0.25 µm) and a Thermo Fisher ISQ LT mass detector (ionization mode: EI; scan time: 1.00 s; scan mass range: 30–300 m/z) were used setting injector and transfer line at 250 and 240 °C, respectively, and a temperature ramp from 60 to 240 °C at 3 °C min^{-1} (carrier gas: He 1 mL min^{-1}). The pure EO was diluted (1:10 v:v) in hexane (95%, Sigma-Aldrich, Munich, Germany), and 0.2 µL were injected at a split ratio of 1:30. The identification of peaks was made using computer matching against the commercial libraries (NIST 05, Wiley FFNSC and ADAMS) comparing linear retention indices (LRI). The LRIs were calculated using the formula of Van den Dool and Kratz (1963) by comparing the retention times of the compounds to be identified with those of a standard mixture of alkanes (C8-C20 saturated alkanes standard mixture, Supelco®, Bellefonte, PA, USA) which was analyzed in GC–MS under the identical operating conditions as the sample (Yoshiro 1976; Davies 1990; Jennings 2012; Adams 2017).

Formulation and physical characterization of nano-emulsions

The *A. sativum* EO-based nano-emulsions were obtained using different methodologies characterized by different amounts of energy supplied to the system (Supplementary Table S1). In detail, we developed four garlic EO-based formulations: (i) Raw emulsion (RAW), (ii) Sonicated nano-emulsion (SN), (iii) Sonicated and Microfluidized nano-emulsion (SH) and (iv) Microfluidized nano-emulsion (HPM). The RAW emulsion was obtained through the self-emulsification method (low-energy process) described by Bouchemal et al. (2004) with modifications. Specifically, to obtain the organic phase, GEO and Tween 80® (polyoxyethylene (20) sorbitan monooleate, Sigma-Aldrich, Munich, Germany) (ratio 3:1 w:w) were mixed for 30 min at 7,000 rpm at room temperature (25 ± 2 °C). Double-distilled water (aqueous phase) was then added slowly (1 mL min^{-1}) to the preliminary mixture (ratio 4:1 w:w) without mixing. The obtained emulsion (EO 15% w/w; Tween 80® 5% w/w; water 80% w/w) was mixed for 3 h at 7,000 RPM. Aliquots of the obtained RAW emulsion were used to develop the other EO-based nano-formulations like SN, SH, and HPM.

The SN nano-emulsion was obtained using the self-emulsifier process followed by sonication (high-energy process) as described by Laudani et al. (2022) with modifications. The RAW emulsion was sonicated for 5 min in an ice bath using a UP200ST ultrasonic immersion homogenizer (Hielsher©, Teltow, Germany) at 100 W power. The (HPM) nano-emulsion was obtained by high-pressure micro-fluidization technique as described by Modafferi et al. (2024), using an LM20 microfluidizer (Microfluidizer™ Processor, USA) for 5 cycles at 30,000 PSI. To avoid the degradation of the EO due to the heat generated during the process, the interaction chamber and heat exchanger were immersed in an ice bath to maintain a low temperature (< 10 °C). The SH nano-emulsion was obtained by integrating both the sonication and the microfluidization processes, respectively, at the same operative conditions. The nano-emulsions were then stored at 4 °C until characterization.

Dynamic Light Scattering (DLS) instrument was used to evaluate the physical characteristics. Particularly, droplet size, polydispersity index (PDI), and surface charge (ζ) values of each developed nano-formulation were measured. The analysis was repeated 1, 7, 50, and 100 days after preparation to assess the stability of the developed nano-emulsions over time. The DLS analyses were done by diluting the samples with double distilled water (1:400 v:v).

Residual contact toxicity against *Planococcus citri*

The bioactivity of the developed garlic EO-based nano-emulsions was determined through leaf dip method to evaluate their residual contact toxicity. Circular sections (Ø 5 cm) of untreated citrus leaves were individually immersed for 10 s in different nano-formulation dilutions. The treated leaf discs were dried at room temperature and placed in ventilated plastic Petri dishes (Ø 5.5 cm). Fifteen unsexed 3rd instar of *P. citri* nymphs from the laboratory rearing were gently placed on the surface of treated leaf. Petri dishes were placed inside climate chambers set at the same climatic conditions used for insect rearing. Mortality was recorded 24 and 48 h after treatment. Insects were considered dead if immobile after stimulation with a fine brush. Preliminary tests were conducted to check mortality at high concentration (2.5% EO). To estimate lethal doses, only formulations that caused 100% mortality of the exposed mealybugs were subsequently tested by applying serial dilutions (1.87, 1.25, 0.93, 0.625, and 0.46% EO). Dilutions of each nano-emulsion were made by adding the required amount of distilled water. Each dose was replicated six times and distilled water was used as untreated control.

Toxicity toward *Apis mellifera*

The acute toxicity of SN, SH, and HPM nano-emulsions toward *A. mellifera* was evaluated using LD_{50} and LD_{90} estimated on *P. citri* after 48 h. The raw emulsion was excluded due to its instability and low efficacy against *P. citri*. For this trial, we followed the topical application method described by Medrzycki et al. (2013). Briefly, bees were collected and anaesthetized with carbon dioxide. Then, 1 µL of nano-emulsions diluted in distilled water was placed on each bee's thorax to achieve the desired concentration. Treated bees were then transferred inside Bugdorm cages (30 × 30 × 30 cm) and fed *ad libitum* with a 50% (w/v) sucrose solution. Each replicate included 10 worker bees and the experiment was replicated six times. The experimental procedure included a treated (dimethoate applied at 0.01 ppm) and an untreated (distilled water) control, and it was conducted under laboratory conditions at 25 ± 1 °C, 70 ± 5% R.H. with a photoperiod of 16 h:8 h (L:D). The mortality of the bees was recorded at 24, 48, and 96 h after the treatment. Specimens were considered dead if they failed to move when stimulated with a fine brush.

Data analysis

Datasets were checked for normality and homoscedasticity of variance through Levene and Shapiro–Wilk tests ($P > 0.05$) and log-transformed whenever needed. Analysis of variance (ANOVA) was used to assess the differences in physical characteristics over time among the different nano-formulations with size, polydispersity index (PDI), and zeta potential values as dependent variables, and the methodology used as fixed factors. Multiple comparisons were assessed by Tukey's HSD post hoc test. *P. citri* mortality registered 24 and 48 h after the treatment was corrected for control mortality using Abbott's formula (Abbott 1925). LD_{50} and LD_{90} values and their fiducial limits were estimated using the Probit analysis. LD values were considered significantly different if their 95% fiducial limits did not overlap. The effects of the different treatments on honeybee mortality were subjected to the Kruskal–Wallis test. Statistics were carried out using IBM® SPSS® Statistics v. 23 (IBM Corp. Released 2015. Armonk, NY, USA).

Results

Chemical composition of *Allium sativum* EO

The chemical composition of *A. sativum* EO is shown in Table 1. A total of 44 peaks were recorded (Supplementary Fig. S1), and twenty-four compounds corresponding to 96.3% of the total area were identified from GC–MS analysis. The *A. sativum* EO was composed of sulfur compounds with diallyl disulfide (37.26%), diallyl trisulfide (28.15%), diallyl tetrasulfide (12.20%), 1-allyl-3-(2-(allylthio)propyl) trisulfane (6.69%) and diallyl sulfide (5.84%) as the most abundant detected compounds.

Physical characteristics of *Allium sativum* EO-based nano-emulsions

The developed nano-emulsions showed different physical characteristics. The use of high-energy methods allowed the development of highly stable nano-emulsions with droplet size in the nanometric range, low PDI values, and good stability indicated by negative surface charge. Conversely, the self-emulsifier method resulted in droplet sizes that did not fall within the nanometric range (> 500 nm) and a high polydispersity index (PDI tending to 1). Statistical differences were recorded over time in all physical characteristics between the different production processes ($P < 0.05$). Generally, the droplet size showed an increasing trend over time. After one day the smallest droplet sizes were registered in SH and HPM methods (72.61 ± 0.13 and 73.56 ± 0.19 nm, respectively) ($F = 12{,}986.2$; df = 3; $P < 0.01$), while at the end of the observations (100 days after the nano-emulsions production), the droplets in the HPM nano-emulsion (108.3 ± 0.35 nm) were smaller than those of the formulations produced with the other methods ($F = 287.81$; df = 3;

Table 1 GC–MS analysis of *A sativum* EO

Component	LRI[a]	LRI[b]	RT[c]	Area (%)
Allyl isopropyl sulfide	825	826	3.2	*
1,2-Dithiolane	841	842	3.44	0.22%
Diallyl sulfide	856	850	3.67	5.84%
Allyl-n-propyl sulfide	871	875	3.9	*
Isopropyl methyl disulfide	895	899	4.27	*
Allyl methyl disulfide	917	922	4.77	1.65%
Dimethyl trisulfide	970	962	6.1	*
1,3-Dithiane	1021	1027	7.57	*
Allyl propyl disulfide	1049	1048	8.54	0.19%
Diallyl disulfide	1082	1082	9.68	37.26%
1-Propenyl propyl, trans disulfide	1094	1100	10.08	0.09%
Methyl 2-propenyl trisulfide	1137	1142	11.77	1.20%
4-Methyl-1,2,3-trithiolane	1151	1150	12.3	1.27%
3-Vinyl-1,2-dithiacyclohex-4-ene	1185	1205	13.68	0.03%
2-Vinyl-4H-1,3-dithiine	1210	1215	14.72	0.11%
4,5-Dimethyl-2-butylthiazole	1226	1226	15.39	*
Allyl isopropil trisulfide	1264	1266	16.97	0.16%
Allyl trisulfide	1301	1296	18.54	28.15%
1,2,3,4-Tetrathiane, 5-methyl-	1357	1359	20.88	0.57%
Diallyl tetrasulfide	1539	1538	28.23	12.20%
4-Ethyl-6-methyl-1,2,3,5-tetrathiolane	1580	1588	29.81	0.03%
1-(1-propenylthio)propyl propyl disulfide	1581	1592	29.87	0.07%
6-Methyl-4,5,8-trithiaundeca-1,10-diene	1591	1591	30.24	0.51%
1-Allyl-3-(2-(allylthio)propyl) trisulfane	1811	1815	38.21	6.69%
Sulfur compounds	96.3%			
Total Identified	96.3%			

[a]Calculated Linear Retention Index

[b]Literature Linear Retention Index

[c]Retention time

* < 0.01%

$P < 0.01$) (Fig. 1A). The PDI registered during the entire period of observation showed low values (0.06 ± 0.006—0.187 ± 0.01) in SN, SH, and HPM nano-emulsions, while the RAW nano-emulsion was characterized by high PDI values (0.912 ± 0.07–0.988 ± 0.008). After one day, statistical differences were observed among all nano-emulsions, and the HPM method highlighted the best homogeneity (0.06 ± 0.006) ($F = 129.1$; df = 3; $P < 0.01$). On the other hand, 100 days after preparation, statistical differences were observed almost among all the different production methods ($F = 5.21$; df = 3; $P < 0.05$) (Fig. 1B). The ζ -potential analysis showed negative values for all the formulations, and the surface charge varied with time and the production method ($P < 0.05$) (Fig. 1C).

Toxicity against *Planococcus citri*

The *A. sativum* EO-based nano-formulations exhibited different efficacy against the immature stages of *P. citri*. The preliminary trial at the high percentage of active ingredient (a.i.) (2.5% of EO) showed that the nano-emulsions obtained through the use of a high-energy process (i.e., SN, SH and HPM) provided effective control of the pest, resulting in 100% mortality. Differently, RAW emulsion did not particularly affect the insects either 24 or 48 h after the treatment (Fig. 2). The mortality registered in *P. citri* treated with serial dilution of SN, SH, and HPM fitted in the Probit model ($P > 0.05$) showing dose–response mortality both 24 and 48 h (Fig. 3). In all cases, the lethal doses (LD_{50} and LD_{90}) highlighted no statistical difference between the different exposure times (24 and 48 h) and between the different production processes (Table 2).

Toxicity toward *Apis mellifera*

The acute topic toxicity of the nano-emulsions (SN, SH, and HPM) toward *A. mellifera* workers is shown in Fig. 4. 96 hours after the exposure, no statistical differences were observed among LD_{50}, LD_{90}, and negative control (water) for all the developed nano-emulsions. Conversely, statistical differences were observed between positive control (dimethoate) and the LD_{50} and LD_{90} of nano-emulsions ($H = 23.000$; df = 7; $P < 0.05$). In particular, the nano-emulsions had no effect on the exposed honeybees (100% survival), while dimethoate resulted in the death of all the bees.

Discussion

The GC–MS analysis showed that garlic EO was mainly composed of sulfur compounds (over 95%), and among them, diallyl disulfide was the most abundant (37.26%) followed by diallyl trisulfide (28.15%). Modafferi et al. (2024) on the other hand showed that garlic EO was mainly composed of diallyl disulfide (73.5%) followed by diallyl sulfide (16.2%). According to our results, Sommano et al. (2016) proved that garlic EO from different countries contained the same proportion of these two principal compounds. However, the amount of these molecules within the EO depends on the extraction and drying methodologies (Satyal et al. 2017; Condurso et al. 2019), as well as by the sampling technique can also impact the identified compounds. As an example, in the present study, garlic EO diluted in hexane was directly injected in GC–MS port; this methodology can impair the identification of the smallest EO components with retention indexes similar to the solvent's one, since their GC_MS pikes can be covered by solvent (i.e., hexane). On the other hand, the methodology used by Modafferi et al.

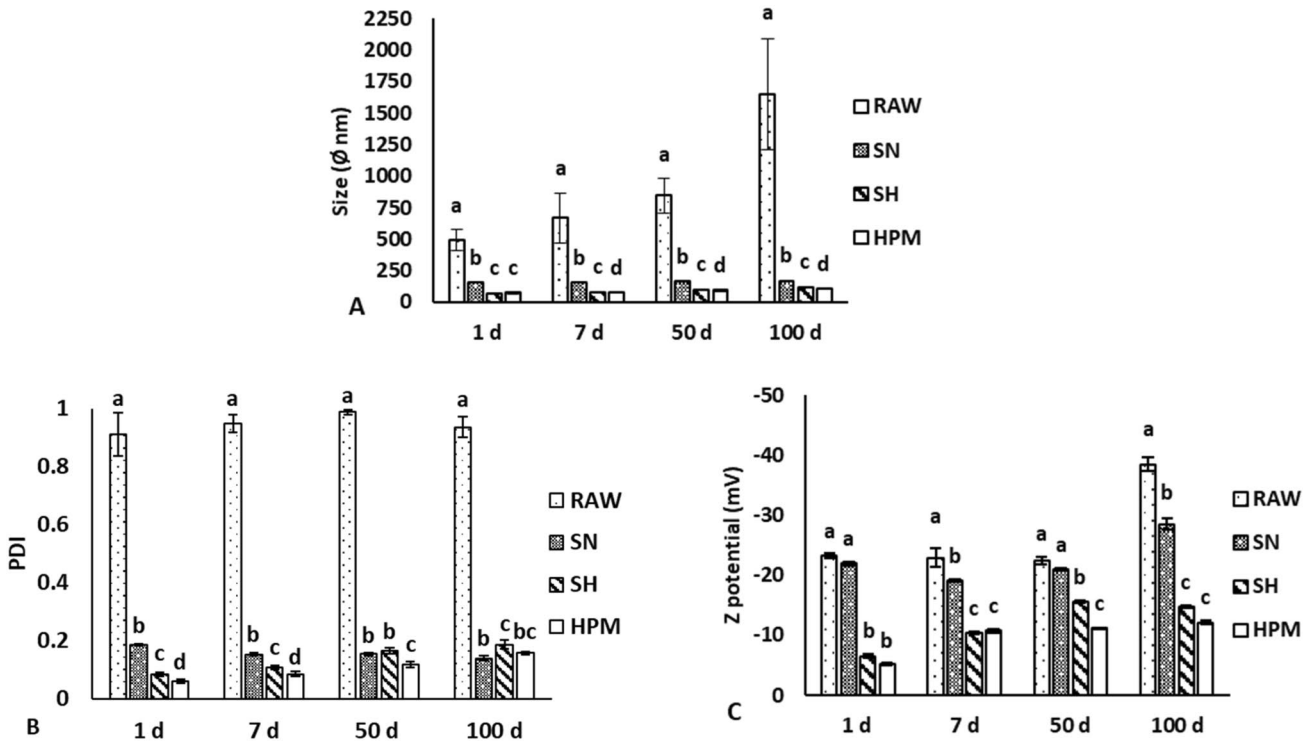

Fig. 1 Physical properties (A = Size; B = PDI; C = ζ-potential) (mean ± SE) of nano-emulsions 1, 7, 50 and 100 days after production. Different letters indicate statistical differences among the methods within the same time (ANOVA, $P < 0.05$)

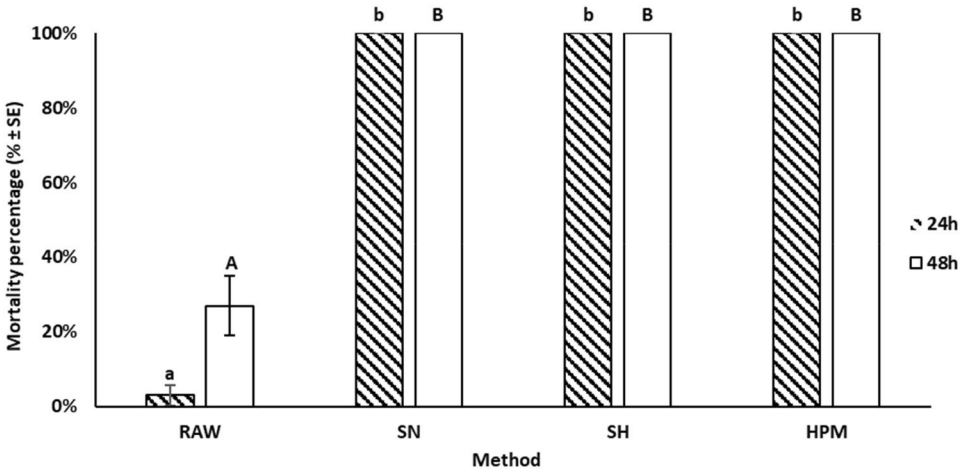

Fig. 2 Mortality (% ± SE) 24 and 48 h after the exposure to *Allium sativum* EO-based nano-emulsions against 3rd instars of *Planoccoccus citri* in the preliminary test (2.5% of EO). Different letters indicate statistical differences among the different methods at the same exposure time (ANOVA, $P < 0.05$)

(2024), i.e., headspace SPME (solid phase micro extraction) technique, requires no solvent but can be less sensitive to the less volatile molecules or to substances weakly affine to the selected fiber or extraction substrates.

This work aimed to develop four different *A. sativum* EO-based insecticide nano-emulsions with high a.i. (15% w/w) and low surfactant (5% w/w) amounts through mixed bottom-up/top-down approaches. Generally, bottom-up processes cannot produce emulsions with fine and homogeneous droplets in the nano-meter range (Campolo et al. 2020). In agreement with this study, our RAW emulsion had mean droplet sizes around 500 ± 83.60 and 1,647 ± 440.69 nm after 1 and 100 days, respectively. Furthermore, the RAW emulsion exhibited highly polydisperse droplets with PDI values close to 1 throughout the observation times. Conversely, several studies reported the effectiveness of these approaches in the preparation of nano-emulsions with other EOs. Using the self-emulsification process, Chang and McClements (2014) obtained a transparent orange EO nano-emulsion (20% surfactant, 10% oil phase, and 70%

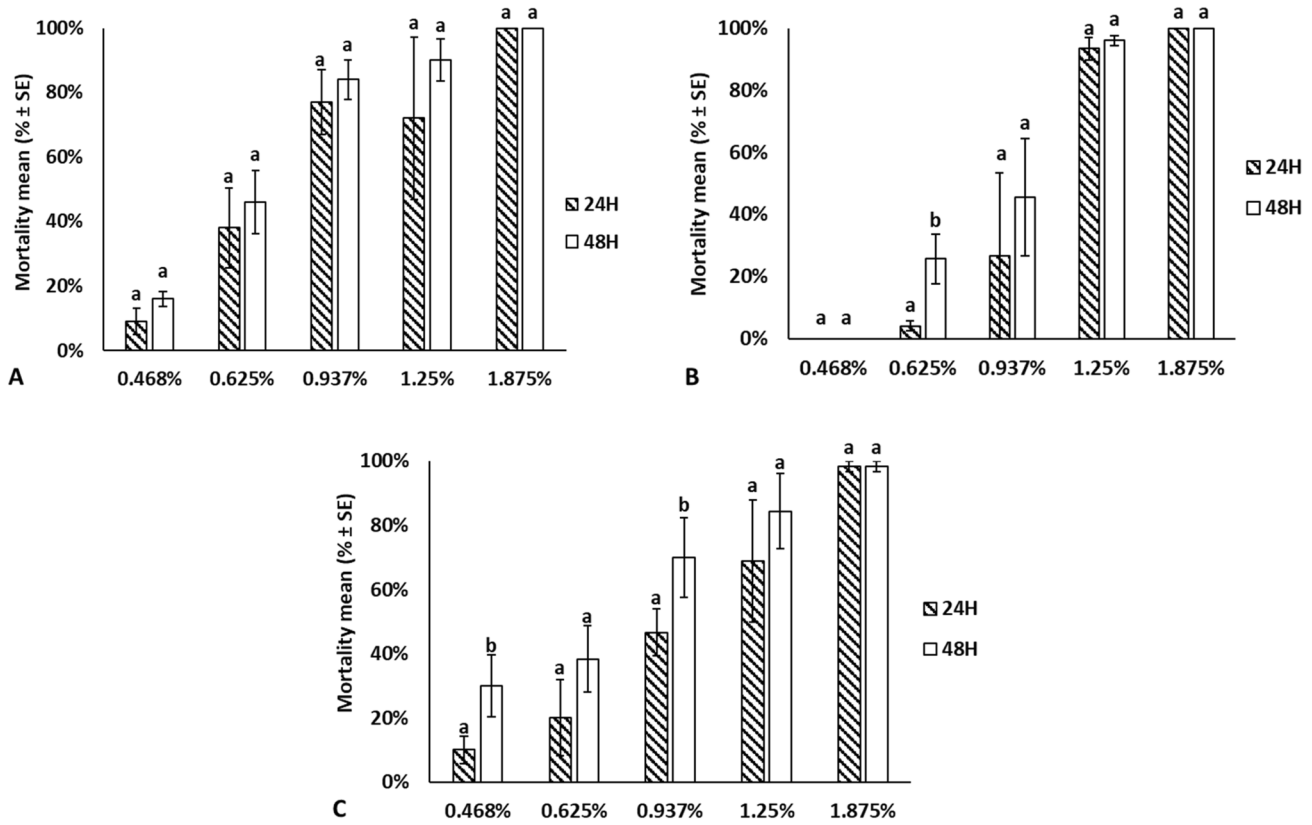

Fig. 3 Dose–response mortality (% ± SE) caused by the developed nano-emulsions (A = SN; B = SH; C = HPM) against 3rd instars of *Planococcus citri* 24 and 48 h after the treatment. Different letters indicate statistical differences between different times of exposure within the same dose (EO %) (ANOVA, $P < 0.05$)

Table 2 Estimated LD_{50} and LD_{90} of developed *Allium sativum* EO-based nano-emulsions against immature stages of *Planococcus citri* 24 and 48 h after the exposure

Formulation type	Time	LD[a]	Estimated	Lower bound	Upper bound	X (df[b])	P level
SN[c]	24 h	50	0.764	0.632	0.900	3.663 (3)	0.300
		90	1.378	1.124	2.054		
	48 h	50	0.653	0.538	0.762	0.562 (3)	0.905
		90	1.104	0.920	1.579		
SH[d]	24 h	50	0.950	0.877	1.029	3.306 (3)	0.653
		90	1.248	1.131	1.499		
	48 h	50	0.826	0.744	0.909	4.996 (3)	0.416
		90	1.211	1.072	1.506		
HPM[e]	24 h	50	0.925	0.783	1.102	1.091 (3)	0.779
		90	1.676	1.346	2.575		
	48 h	50	0.684	0.529	0.825	0.503 (3)	0.903
		90	1.407	1.108	2.363		

Values were considered significantly different if their 95% fiducial limits did not overlap

[a]Lethal dose

[b]Degrees of freedom

[c]Sonicated nano-emulsion

[d]Sonicated + Microfluidized nano-emulsion

[e]Microfluidized nano-emulsion

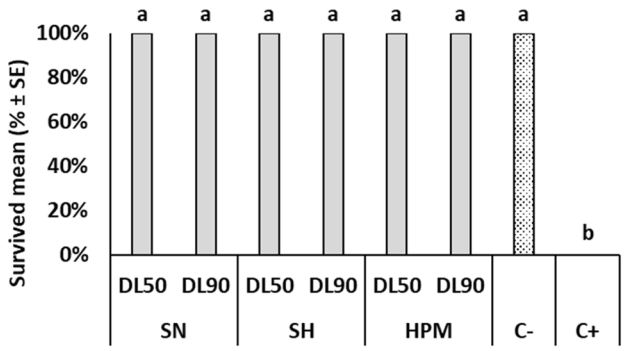

Fig. 4 Percentage of survived *Apis mellifera* workers 96 h after exposure to nano-emulsions (SN, SH and HPM), negative control (water) and positive control (dimethoate). Different letters indicate statistical differences among the different treatments (Kruskal-Wallis test, $P < 0.05$)

water w/w) that showed droplet size around 20 nm. Another study reported that the phase inversion temperature (PIT) method allowed to obtain different *Origanum vulgare* EO-based nano-emulsions (20% surfactant; 10% oil phase and 70% water w/w) with droplet size in the range of 35–55 nm (Moraes-Lovison et al. 2017). The use of the bottom-up process presents some limitations depending on the type of EO, the surfactant, and their relative ratios that reduce its application in nano-emulsion preparation (Sessa and Donsì 2015; Donsì and Ferrari 2016). This aspect should be considered since high amount of surfactant often results in phytotoxicity toward crop plants (Temple and Hilton 1963; Falk et al. 1994; Appah et al. 2020; Mirgorodskaya et al. 2020).

The top-down approaches allowed us to develop garlic EO-based nano-emulsions (i.e., SN, SH, and HPM) with droplet size in the nanometric range (< 200 nm) and good homogeneity (PDI less than < 0.2), Other researchers highlighted the efficacy of the top-down approaches to develop garlic EO-based nano-emulsions. Through sonication methods, Palermo et al. (2021) prepared several EOs-based nano-insecticides, including a garlic nano-emulsion. This garlic formulation had similar characteristics to our SN nano-emulsion, with a droplet size of 144.30 ± 0.15 nm and PDI of 0.164 ± 0.008. Similarly, Liu et al. (2022) developed different garlic EO-based nano-emulsions using different sonication duration (0, 1, 5 and 10 min). All the developed formulations had droplet sizes and PDI values higher than those obtained in this study by SN, SH, and HPM approaches.

The surface charge, achieved (− 5.13 to − 38.5 mV) in our garlic EO nano-emulsion should contribute, together with the steric repulsion, to the stability recorded over time. Usually, surface charges around ± 30 mV are considered predictors of stability, but the use of non-ionic surfactants (i.e., Tween 80) owes their main stability to both steric and electrostatic repulsions (Gul et al. 2018; Akbari and Nour 2018; Liu et al. 2022). In these cases, ζ-potential values of ± 20 mV coupled with small droplet size could stabilize nano-emulsions (Müller et al. 2001; Malhotra and Coupland 2004; Campolo et al. 2020).

The biological activity of aqueous or solvent garlic extracts against scale pests has been quite well studied. As an example, Fand et al (2012) demonstrated the efficacy of aqueous garlic extract against 2nd instar nymphs of *Phenacoccus solenopsis* Tinsley (Hemiptera: Pseudococcidae), proving that the topical application of a 1% concentrated garlic solution reduced the insect population by about 50%. Similarly, Prishanthini and Vinobaba (2014) highlighted the efficacy of different botanical extracts, including garlic, against *P. solenopsis* adults. The results showed a dose–response mortality with an estimated LC_{50} of 1.15% for *A. sativum* extract. Garlic extract was also found effective against *Aulacaspis tubercularis* Newstead (Hemiptera: Diaspididae), and its application reduced the insect population in field conditions (Siam and Othman 2020). Furthermore, garlic extract exhibited strong residual toxicity (LC_{90} = 121.96 ppm) against *Icerya purchasi* Maskell (Homoptera: Margarodidae) (Allam et al. 2022). Other authors reported that the methanolic extract and EO of garlic were effective against 3rd instar of *Pseudococcus viburni* Sigornet (Hemiptera: Pseudococcidae), with an estimated LC_{50} of 0.12% and 0.31% 48 h after the exposure, respectively (Ramzi et al. 2022), and against *Pseudococcus longispinus* Targioni Tozzetti (Hemiptera: Pseudococcidae) with an estimated LC_{50} of 1.65% (Smith 2015).

Limited studies are available about the EO obtained from this plant toward scale pests. Mwanauta et al. (2023) reported the efficacy of this EO against *Paracoccus marginatus* Williams and Granara de Willink (Hemiptera: Pseudococcidae). The results obtained 72 h after the exposure highlighted a good mortality (73.0 ± 1.7%) caused by pure garlic EO at 1.5% concentration. Modafferi et al. (2024), also investigated the bioactivity of garlic EO-based nano-emulsion against *P. citri*. The results highlighted a high efficacy of this formulation through direct and indirect application of EO exhibiting LC_{90} of 0.967 and 1.088%, respectively, 48 h after the exposure. The LD_{90} calculated in this study after 24 and 48 h are comparable to the above-mentioned results and suggest that the formulation of the EO in nano-emulsion can improve the bioavailability and bioactivity of garlic EO also against other target scale pests.

The selectivity of these substances toward non-target organisms (e.g., predators, parasitoids, and pollinators) is a poorly investigated aspect about the use of EOs or their formulations as biopesticides. Nevertheless, several studies have proven the detrimental effect of biopesticides toward several bee species, including *A. mellifera* (reviewed in Borges et al. 2021; Cappa et al. 2022). Concerning botanicals, EOs were extensively investigated for the potential use in beekeeping for controlling *Varroa* spp. mites or other

parasites on *A. mellifera*, and the majority of these compounds showed low toxic effects for honeybees (Ntalli et al. 2022; Catania et al. 2023). The present study tested the garlic EO-based nano-emulsions (i.e., SN, SH, and HPM) for selectivity toward *A. mellifera*. These nano-emulsions did not affect the honeybee mortality, and 100% of the specimens survived after exposure to all the concentrations of the tested formulations. Overall, the selectivity of these natural substances was influenced by some variables (e.g., type of EO, application rate, doses, insect species, etc.) (Giunti et al. 2022). For example, Xavier et al. (2015) assessed the toxicity of several botanical pesticides toward *A. mellifera* adults. The results demonstrated that citronella oil, eucalyptus oil, garlic extract, neem oil, and rotenone were highly toxic against bee larvae in ingestion bioassay, while andiroba oil was not statistically different from negative control. Furthermore, all the tested botanical extracts were repellent toward adult bees (Xavier et al. 2015). Some EOs can cause severe mortality for honeybees, as well as for other bee species. Melo et al. (2018) proved that the topical application of thymol and carvacrol caused high mortality (> 80%) toward *A. mellifera* adults, as also reported in *Tetragonisca angustula* (Latreille) (Meliponinae), in which more than 70% of mortality was caused by topical application of *Artemisia annua* L. EO (Seixas et al. 2018). Similarly, da Silva et al. (2020) reported lower toxicity of mint and ginger EOs toward honeybees than oregano and thyme EOs. Nevertheless, toxicological studies should take into account that thyme EOs is considered safe for honeybees in field conditions, since it is a widely used acaricide for the control of *Varroa* mites (van der Steen and Vejsnæs 2021). Moreover, EO doses and its application methods used for toxicological test against *A. mellifera* should be comparable to those used in real conditions. As an example, garlic extract and EO were safely applied on honeybee colonies by Mazeed and El-Solimany (2020) to manage *Varroa destructor* Anderson & Trueman.

Conclusion

In conclusion, this study is focused on developing garlic essential oil (GEO)-based nano-emulsions for potential insecticidal applications. Mixed and single bottom-up/top-down approaches were used to develop the nano-emulsions, the latter proving effective in achieving nanometric droplet sizes (< 200 nm) and good homogeneity. The limited research on their bioactivity against *P. citri* prompted this investigation, which confirmed promising results in terms of mortality. An essential aspect addressed in this study was the selectivity of garlic EO-based nano-emulsions for honeybee workers. The results indicated a lack of adverse effects of garlic EO on *A. mellifera* mortality, suggesting its environmentally friendly potential. Further research is warranted to explore the full potentiality of these nano-emulsions in integrated pest management strategies and their broader ecological impact.

Author contribution

AM, GG and OC conceived and designed research. AM, FL and IL conducted experiments. AM, AU, MPH, MR and VP analyzed data. AM wrote the manuscript. All authors revised, read and approved the manuscript.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s10340-024-01800-2.

Funding Open access funding provided by Università degli Studi di Salerno within the CRUI-CARE Agreement. This study was carried out within the project ASTER from Call Prima Sect. 2 2021—Multi-Topic, the Agritech National Research Center and received funding from the European Union Next-Generation EU (PIANO NAZIONALE DI RIPRESA E RESILIENZA (PNRR) – MISSIONE 4 COMPONENTE 2, INVESTIMENTO 1.4 – D.D. 1032 17/06/2022, CN00000022). This manuscript reflects only the authors' views and opinions, neither the European Union nor the European Commission can be considered responsible for them.

Data availability The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Conflict of interest The authors have no relevant financial or non-financial interests to disclose.

Ethical approval Not applicable.

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CHAPTER 5

Bioactivity of *Allium sativum* essential oil-based nano-emulsion against *Planococcus citri* and its predator *Cryptolaemus montrouzieri*

(This is the published version in

Industrial Crops & Products

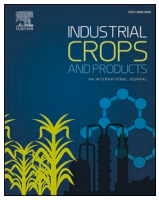
https://doi.org/10.1016/j.indcrop.2023.117837)

Industrial Crops & Products 208 (2024) 117837

Contents lists available at ScienceDirect

Industrial Crops & Products

journal homepage: www.elsevier.com/locate/indcrop



Bioactivity of *Allium sativum* essential oil-based nano-emulsion against *Planococcus citri* and its predator *Cryptolaemus montrouzieri*

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ARTICLE INFO

Keywords:
Biopesticide
Citrus mealybug
Garlic
High-pressure microfluidizer
Insecticide
Side Effects

ABSTRACT

Botanical extracts, in particular essential oils (EOs), could be the ideal candidates for the development of biopesticides as an alternative to synthetic pesticides. However, some limitations of EOs (high flammability, volatility, degradability, poor solubility in water) prevent their use under real operational conditions. Nanotechnologies are useful tools to overcome the above-mentioned limitations of these natural substances. Furthermore, encapsulation in nano-delivery systems (nanoparticles and nano-emulsions) can improve the functional properties of EOs. In this context, this study aimed to develop a highly stable, concentrated garlic nano-emulsion (15%) and to evaluate the acute toxicity with different exposure routes towards *Planococcus citri* and its predator *Cryptolaemus montrouzieri*. First, garlic EO was used to develop a nano-emulsion (15% EO; 5% Tween 80; 80% water) using a high-pressure microfluidizer; then both the crude EO and EO in nano-emulsion were chemically investigated by Solid Phase Microextraction-Gas Chromatography-Mass Spectrometry (SPME-GC-MS) technique. The nano-emulsion was physically characterized by dynamic light scattering analysis over time (24 h, 3 months and 1 year after preparation) and used in bioassays involving both the target and non-target organisms. Results showed that the garlic EO consisted of over 95% sulphur compounds with diallyl disulphide as the most abundant component, and the developed nano-emulsion remained stable even after 1 year, with droplets' dimension within the nanometric range (221.4 nm). The nano-formulation was effective against the target pest after 48 h from the treatment (Direct: $LC_{90} = 0.967\%$; Indirect: $LC_{90} = 1.088\%$), while it had no effect on *C. montrouzieri*. These promising results highlight the potential of garlic-based nano-emulsion as effective and environmentally friendly insecticide for pest control.

1. Introduction

Planococcus citri Risso (Hemiptera: Pseudococcidae) is a serious pest that feeds on a variety of economically important crops and greenhouse ornamentals (Afifi et al., 2010). This mealybug is present in all citrus-growing regions worldwide, and its infestations can cause consistent production losses. Additionally, the abundant honeydew excreted by *P. citri* promotes the development of sooty mould and attracts other undesirable insects such as ants (Mansour et al., 2018; Herrick et al., 2019). Among the several strategies used for the control of this pest, the majority relies on the use of chemical pesticides, despite the biological control approach can ensure optimal control while limiting the negative impacts of synthetic pesticides (Abdollahdokht et al., 2022; Laudani et al., 2022). Microbial biopesticides (i.e. entomopathogenic fungi) and augmentative biological control applications, such as the parasitoid *Leptomastix dactylopii* (Howard) (Hymenoptera: Encyrtidae) or the predator *Cryptolaemus montrouzieri* (Mulsant) (Coleoptera: Coccinellidae), are commonly used in the most important citrus-producing countries (Zappalà, 2010; Ghaffari et al., 2017; Mansour et al., 2018). The awareness of the negative effects of synthetic pesticides on the environment and human health, and the resulting growing demand for pesticide-free food products has prompted researchers to find new eco-friendly control methods (Giunti et al., 2019). Among botanical extracts, which are considered alternative solutions to

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https://doi.org/10.1016/j.indcrop.2023.117837
Received 3 October 2023; Received in revised form 15 November 2023; Accepted 16 November 2023
Available online 28 November 2023

conventional pesticides, plant essential oils (EOs) represent an interesting challenge for the development of new biopesticides. EOs are composed of a wide variety of substances (e.g. terpenoids, aromatic compounds, etc) which are produced by several plant families for different purposes such as plant defence and signalling to other organisms (Burt, 2004; Bakkali et al., 2008; Campolo, 2022). In the past decades, there has been a growing research interest in these extracts as potential tools for controlling various pests, as EOs are known to affect the physiological, biochemical, and metabolic activities of insects and plants (Priestley et al., 2003; Zhou et al., 2008; Ben Abdallah et al., 2023; Duque et al., 2023; Ricupero et al., 2023).

Allium sativum Linnaeus (Amaryllidaceae), commonly known as garlic, is a globally cultivated crop primarily used for food spicy and medical purposes. The promising toxicity of garlic extracts has been demonstrated against several agricultural pests (Palermo et al., 2021; Ricupero et al., 2022) including insect mealybugs (Hemiptera: Pseudococcidae) such as *P. citri* (Cloyd et al., 2009; Mwanauta et al., 2021; Ramzi et al., 2022). Despite the use of garlic as a plant protection product poses no risk to consumers (Anastassiadou et al., 2020), the non-target toxicity of garlic EO on beneficial arthropods has been merely documented and it requires investigation to implement IPM programs (Ricupero et al., 2022; Giunti et al., 2022a). However, EOs have intrinsic properties that make their use problematic under real operating conditions. High volatility, phytotoxicity, poor solubility in water, rapid degradation and high flammability are the main issues that must be addressed before using EOs as pest control tools (Campolo et al., 2020).

The development of stable and effective insecticide formulations is, therefore, necessary to transfer laboratory results into field applications. This transition can be accelerated by nanotechnologies which can help to overcome or mitigate these constraints. Furthermore, nanotechnologies can enhance the biological and functional properties of EOs by allowing a gradual release of the active ingredients, improving target surface coverage and enhancing bioactivity (Campolo et al., 2017; Pavoni et al., 2020; Sciortino et al., 2021). In this context, nano-emulsion and nano-encapsulation of EOs appear to be the most promising approaches for developing new and eco-friendly EOs-based insecticides. Nano-emulsions (NEs) are dispersed heterogeneous systems, consisting of two immiscible liquids stabilized by an emulsifier. There are two types of NEs: water in oil (W/O) and oil in water (O/W) depending on the dispersing phase (oil or water respectively). Nano-emulsions can be produced using low-energy methods (e.g., composition and temperature phase inversion; high speed homogenization) or high-energy methods (e.g., sonication, high-pressure homogenization, high-pressure micro-fluidization) (Donsì and Ferrari, 2016). High-energy methods generate more stable nano-formulations with optimal compositions, as they enable the use of higher concentrations of the active ingredients (a.i.) and require smaller quantities of surfactants compared to low-energy methods (McClements and Rao, 2011; Gurpreet and Singh, 2018). Additionally, obtaining time-stable nano-emulsions relies on understanding the chemical composition of EOs and selecting the appropriate surfactants, which depends on the hydrophilic-lipophilic balance (HLB) of EOs (Campolo et al., 2020).

The main objective of this study was the development of a stable garlic EO-based nano-emulsion using high-pressure micro-fluidization technique to include a high amount of a.i. in the nano-emulsion while ensuring its stability over time. Both chemical and physical characterization of garlic EO and the nano-emulsion were assessed by Solid Phase Microextraction-gas chromatography-Mass Spectrometry (SPME-GC-MS) and dynamic light scattering (DLS) analyses. Additionally, the target and non-target insecticidal activity of the developed formulation was evaluated towards *P. citri* and its main predator *C. montrouzieri*, respectively.

2. Materials and methods

2.1. Insect rearing

Planococcus citri was reared for several generations at the entomology laboratories of the Department of Agriculture, University *Mediterranea* of Reggio Calabria, Reggio Calabria, Italy. The original insect colony was collected in 2020 in an organic citrus orchard located in Reggio Calabria. Insects were reared for several generations on butternut pumpkin fruit inside a climatic chamber under constant climatic conditions: 28 ± 1 °C, 70 ± 5% R.H., with a photoperiod of 12:12 h (L:D).

Cryptolaemus montrouzieri specimens were provided by the biofactory "Biofabbrica Insetti Utili – Ente Sviluppo Agricolo, Regione Siciliana" (Ramacca, Italy) where the coccinellids were fed upon *P. citri* infested potato sprouts. For the experiment, newly emerged *C. montrouzieri* adults (1d-old) were obtained from same aged pupae previously isolated in a ventilated plastic box. Adults of *C. montrouzieri* were thus sexed under a stereomicroscope and isolated in each group of 5 females and 5 males in ventilated plastic tubes. Coccinellid beetles were fed with an honey-based jelly food (Ricupero et al., 2020) and kept at the aforementioned laboratory conditions until the beginning of the bioassay.

2.2. SPME-GC-MS chemical composition of the Garlic-EO and Garlic EO nano-emulsion

To describe the volatile chemical profile of garlic EO and garlic EO nano-emulsion, SPME-GC-MS technique was used.

Approximately 2 mL of each sample were individually placed into a 7 mL glass vial with polytetrafluoroethylene (PTFE)-coated silicone septum. Before the sampling, a thermostatic bath with constant magnetic stirring was used for 15 min to reach thermal equilibrium. The extraction and capture of volatile compounds was performed by using a SPME device from Supelco (Bellefonte, PA, USA) equipped with a 1 cm fiber coated with 50/30 μm DVB/CAR/PDMS (divinylbenzene/carboxen/polydimethylsiloxane). Initially, the fiber was conditioned at 270 °C for 30 min and then it was inserted into the vials and exposed to the headspace for 10 min at 50 °C. Lastly, the SPME fiber was inserted to the GC injector port set to 250 °C in splitless mode for the desorption of the adsorbed components.

The headspace analyses of samples were carried out using a Clarus 500 model Perkin Elmer (Waltham, MA, USA) gas chromatograph equipped with a FID (flame ionization detector) and coupled with a mass spectrometer (Rizzo et al., 2023). The capillary column used for the separation of compounds was a Varian Factor Four VF-1. The operative chromatographic and spectrometric conditions were as follows: the oven GC temperature program was as follows: started from 50 °C then ramped up to 220 °C at a rate of 6 °C/min and isothermal at 220 °C for 20 min. Helium was used as carrier gas at flow of 1.0 mL min^{-1} in constant mode. The mass spectra were obtained in the electron impact mode (EI), at 70 eV, in full-scan mode in the range 30–450 *m/z*. For the identification of compounds, the matching between their mass spectra with those stored in the Nist 02 mass spectra library database, was performed. Further, the linear retention indices (LRIs), were calculated using a series of alkane standards (C_8–C_{25} n-alkanes) and compared with those available in the literature. The relative concentration of each identified compound, expressed as percentage, was calculated by normalizing the peak area over the total area of all identified peaks in the chromatogram without the use of an internal standard and any factor correction.

2.3. Nano-emulsion formulation and characterization

Food grade *A. sativum* (thereafter Garlic) EO extracted from bulbs was purchased by Esperis s.p.a. (Milan, Italy) (Batch No. OL.ES. 4 20/21). The garlic EO nano-emulsion was prepared using the high-pressure

micro-fluidisation technique. Firstly, to prepare the organic phase, a solution of EO and Tween 80® (Polyoxyethylene (20) sorbitan monooleate, Sigma Aldrich, Munich, Germany) was mixed using a magnetic stirrer (5 min at 6000 RPM). Then, double-distilled water was added slowly (1 mL min^{-1}) to the organic phase to obtain a pre-emulsion (EO 15% w/w; Tween 80® 5% w/w; Water 80% w/w). The obtained raw emulsion was mixed for 5 min at 7000 RPM. The mixture was then homogenized by a high-pressure microfluidizer (LM20 Microfluidizer™ Processor, USA) at 30,000 PSI. To obtain a homogeneous formulation, the homogenization process was repeated five times. To avoid EO degradation due to heat generated during homogenization, the interaction chamber was coupled with a heat exchanger immersed in an ice bath. In this way, the developed formulation stayed at T < 10 °C. The obtained nano-emulsion was stored in aluminium containers and the bioassays were carried out within two weeks.

The physical characteristics of the garlic EO nano-emulsion were analysed by a Dynamic Light Scattering (DLS) apparatus (Zetasizer Nano, Malvern®). In detail, the droplet dimension (Z-average size), the polydispersity index (PDI), and the droplet surface charge, (ζ-potential) values were measured at 24 h, 3 months and 1 year after the nano-emulsion preparation that was kept at room temperature (25 ± 2 °C). Measurements of the physical characteristics were carried out by diluting the obtained nano-emulsion with double distilled water in order to reach a ratio of 1:200 (v:v); 1 mL and 0.75 mL of diluted nano-emulsions were used to assess the size and the surface charge, respectively.

2.4. Insecticidal activity against P. citri

The insecticidal activity of the developed nano-formulation was evaluated against the 3rd instars of *P. citri*. The application rates were based on preliminary investigations aimed at assessing the minimum dose necessary to cause the total mortality of the tested individuals and the maximum dose that does not significantly affect the mortality of the treated insects in comparison to the untreated control. All the experiments were carried out under laboratory conditions at 28 ± 1 °C, 70 ± 5% R.H. with a photoperiod of 12:12 h (L:D). The citrus leaves used in all the experiments were obtained from an organic orchard where no insecticide treatments were applied one year before the collection. Mortality was assessed 24 and 48 h after treatment. Insects were deemed dead if they did not move their body or were unable to walk. Six replicates (each bearing 15 *P. citri* specimens) were performed for all the different dilutions, while double-distilled water was used as untreated control.

To assess the efficacy of garlic EO nano-emulsion, two distinct experiments were carried out to evaluate the effects of direct exposure to the target pest and the residual activity of the formulated product. These methodologies were designed to simulate the real-operating scenarios in the field where not all insects are immediately affected by the insecticide treatment.

The toxicity of the nano-emulsion upon direct contact with the target pest was assessed using a handle sprayer (2 L Dea, Volpi, Italy). Five serial dilutions of the nano-emulsion, namely 1.25%, 0.625%, 0.31%, 0.156%, and 0.078% of EO concentration in the insecticide formulation were prepared by adding the required volume of double-distilled water (Giunti et al., 2022b). The insects were gently placed on filter paper inside plastic Petri dishes (9 cm in diameter) and sprayed with the Garlic nano-emulsion at the different application rates before reported. Subsequently, the treated insects were kept inside the Petri dishes for 15 min and then carefully placed onto non-treated leaf surfaces fixed inside Petri dishes (5.5 cm in diameter). The trial was carried out under the same environmental conditions used for insect rearing.

The second experiment aimed to evaluate the residual contact toxicity of the formulated EO was carried out using the leaf-dip method to treat the leaf surfaces. Specifically, five serial dilutions of the nano-emulsion were prepared by adding double-distilled water (1.06%, 0.9375%, 0.78%, 0.625%, and 0.53% of EO concentration in the nano-emulsion). Circular sections of citrus leaves (5 cm in diameter) were individually immersed in the desired dilutions of the nano-emulsion for 15 s and then left to dry at room temperature and placed inside a plastic Petri dish (5.5 cm in diameter). Subsequently, 15 unsexed specimens of *P. citri* 3rd instar were gently placed on the treated leaf surface. The Petri dishes were then placed inside climatic chambers set at the previously described environmental conditions.

2.5. Lethal effect on C. montrouzieri

The acute toxicity on *C. montrouzieri* was assessed through topical application of garlic EO nano-emulsion at LC_{90} estimated for *P. citri* (i.e., the concentration of active ingredient able to control the 90% of the tested pest population, see Result Section 3.4, LC_{90} =1.18%) and the maximum dose (MD) applied on pest direct exposure experiment after 24 h (see 2.4 above, MD=1.25%). Per each replicate, five couples (5 females and 5 males) of coetaneous *C. montrouzieri* adults were topically sprayed with the nano-emulsion using a hand sprayer according to the methodology described in (Passos et al., 2022). Thus, predators were moved in a cup arena and their survival was assessed every day for three days after exposure. Predatory coccinellids were considered dead when they did not react after being touched with a paintbrush. Untreated control was also included by spraying *C. mountrouzieri* with double distilled water. The experiment was replicated ten times per each concentration and the untreated control. Also these experiments were carried out under laboratory conditions at 28 ± 1 °C, 70 ± 5% R.H. with a photoperiod of 12 h:12 h (L:D).

2.6. Statistical analysis

The changes in the physical characteristics of the developed formulation over time were analysed using a one-way analysis of variance (ANOVA), with time after formulation as the fixed factor and size, PDI, and zeta potential values as dependent variables. All data met the assumptions required by parametric tests, including normality and homoscedasticity of variance ($p > 0.05$).

Mortality data were corrected for control mortality using Abbott's formula (Abbott, 1925). The concentration-mortality response was evaluated by Probit analysis, in which the median Lethal Concentration (LC_{50}) and LC_{90} values and their fiducial limits were estimated using the results obtained 24 and 48 h after the treatment. LC values were considered significantly different if their 95% fiducial limits did not overlap. The effect of garlic nano-emulsion at the two tested concentrations on the survival of *C. montrouzieri* was analysed using a one-way analysis of variance (ANOVA). Multiple comparisons were evaluated by Duncan's posthoc test. Statistics were conducted on IBM® SPSS® Statistics v. 23 (IBM Corp. Released 2015. Armonk, NY, USA).

3. Results

3.1. Chemical composition

By GC-MS analysis, fifteen compounds in total were identified and listed in Table 1. The chemical composition of the EO in nano-emulsion resembled the composition of crude EO, both from a qualitative point of view as well as in the percentage trend of the detected compounds (Table 1). The major compounds were diallyl disulfide (73.5%; 79.0%) and diallyl sulfide (16.2%; 9.3%), followed by diallyl trisulfide (2.7%, 4.7%), allyl methyl disulfide (3.0%; 2.0%), 1,2-dithiole (1.1%; 1.8%) and allyl methyl trisulfide (0.9%; 1.0%) in crude and nano-formulated EO, respectively. The other sulphur compounds which characterized the two samples did not reach percentages equal to or greater than 1% (i.e., from 0.1% to 0.9%). The chromatograms are reported in Figs. 1 and 2.

Table 1
Chemical composition (mean percentage ± SD) of garlic EO and garlic EO in nano-emulsion as determined by SPME-GC-MS.

N°	COMPONENT[a]	LRI[b]	LRI[c]	EO	Nano-EO
1	allyl chloride	511	516	0.1 ± 0.00	-
2	thiirane, methyl-	645	650*	0.9 ± 0.03	0.9 ± 0.03
3	allyl methyl sulfide	680	678	0.6 ± 0.02	0.1 ± 0.00
4	diallyl sulfide	855	850	16.2 ± 0.09	9.3 ± 0.06
5	allyl methyl disulfide	904	902	3.0 ± 0.02	2.0 ± 0.04
6	1,2-dithiole	938	936	1.1 ± 0.03	1.8 ± 0.06
7	diallyl disulfide	1082	1078	73.5 ± 0.55	79.0 ± 0.60
8	allyl methyl trisulfide	1135	1130	0.9 ± 0.03	1.0 ± 0.05
9	1,2,3-trithiolane, 4-methyl-	1178	1185	0.1 ± 0.00	tr
10	3-vinyl-1,2-dithiacyclohex-4-ene	1193	1190.9	0.2 ± 0.01	0.2 ± 0.01
11	2-vinyl-4 H-1,3-dithiine	1198	1199	0.2 ± 0.01	0.3 ± 0.01
12	cis-geraniol	1240	1236	tr	0.1 ± 0.00
13	diallyl trisulfide	1281	1275	2.7 ± 0.04	4.7 ± 0.05
14	1,2,3,4-tetrathiane, 5-methyl-	1373	1369.3	0.4 ± 0.02	0.5 ± 0.02
15	disulfide, 1-methyl-2-(2-propenylthio) ethyl 2-propenyl	1587	1591*	tr	-
	SUM			99.9	99.9

EO: Percentage mean values of garlic essential oil components; Nano-EO: Percentage mean values of garlic essential oil in nano-emulsion components; tr: (mean value <0.1%); - Not detected.
* Normal alkane RI.
[a] The components are reported according to their elution order on apolar column;
[b] Linear Retention Indices measured on apolar column;
[c] Linear Retention indices from literature;

3.2. Physical characterization

The developed nano-formulation exhibited particle sizes in the nanometric scale, as presented in Table 2. Specifically, the mean size of the nano-emulsion increased significantly (F= 8465.93; df= 2; $p < 0.001$) over time. After 24 h, the particle size was 64.5 ± 0.51 nm, which increased to 82.8 ± 0.45 nm three months later and reached 221.4 ± 2.67 nm after one year. The homogeneity of the developed formulation over time was characterized by low values of PDI, which statistically varied (F= 19.14; df= 2; $p < 0.05$) with the elapsing time. The freshly prepared nano-emulsion had a very low PDI of 0.05 ± 0.01, indicating a monodisperse distribution of particle size. However, three months after the formulation, the PDI increased to 0.12 ± 0.025 and reached the value of 0.17 ± 0.02 after one year. Furthermore, the zeta potential statistically decreased (F= 210.86; df= 2; $p < 0.001$) from − 17.6 ± 1.25 mV after 24 h and reached the minimum value (−30.4 mV) after one year.

3.3. Insecticidal activity against P. citri

The developed nano-formulation exhibited good efficacy against the immature stages of *P. citri* in both direct and residual contact toxicity tests. The experimental data collected at 24 and 48 h after treatments for both direct and residual contact toxicity were analysed using the Probit model, and the results showed good model fit ($p > 0.05$). The LC_{50} values estimated after 24 and 48 h did not show statistical differences within the same group, but comparisons between the LC_{50} values estimated for direct and residual contact toxicity showed significant differences (with non-overlapping fiducial limits). Specifically, after 24 h, the estimated LC_{50} values were 0.363% for direct contact toxicity ($\chi^2 = 6.642$; df= 3; $p = 0.249$) and 0.852% for residual contact toxicity ($\chi^2 = 1.758$; df= 3; $p = 0.624$). After 48 h, the estimated LC_{50} values were 0.248% for direct contact toxicity ($\chi^2 = 5.805$; df= 3; $p = 0.326$) and 0.782% for residual contact toxicity ($\chi^2 = 0.993$; df= 3; $p = 0.803$). The LC_{90} values did not show significant statistical differences for both direct and residual contact toxicity, at both 24 and 48 h after treatment (Table 3).

3.4. Lethal effect on C. montrouzieri

The impact of the developed formulation towards the non-target organism *C. montrouzieri* is depicted in the Fig. 3. At the end of the observations the number of specimens survived to the different treatments was homogeneous among the different groups (F= 1.90; df= 2; $p = 0.172$). In the control treatments no mortality was registered during the trials while the 84.44% ± 6.7 and the 90% ± 5.37 of the exposed beetles survived to the maximum dose tested and LC_{90} estimated for *P. citri,* respectively.

Both males and females of the coccinellid were not affected by the garlic nano-emulsion applied at the LC_{90} and maximum dose (females: F= 1.83; df= 2; $p = 0.182$: males: F= 1.134; df= 2; $p = 0.339$).

4. Discussion

The main objective of this study was to develop an *A. sativum* EO-based nano-emulsion, characterised by a high active ingredient concentration (15%), for the control of the mealybug *P. citri*. The chemical characterization of garlic EO revealed that the main compound was diallyl disulphide followed by diallyl sulfide. According to our results, diallyl disulfide has been reported to be the major compound also in previous research (Mossa et al., 2018). On the other hand, some reports have described GC-MS profiles in which diallyl disulfide had lower relative concentrations than diallyl trisulfide (Herrera-Calderon et al., 2021) or dimethyl trisulfide (Plata-Rueda et al., 2017). However, Satyal et al. (2017) showed how the percentage levels of this compound can also vary depending on the method used to extract the EO.

The physical characteristics of the developed insecticidal formulation indicated a good quality both in terms of size and PDI, as well as of chemical composition stability. Indeed, the freshly produced EO formulation had particle size below 65 nm and a PDI near to zero. The use of microfluidizer apparatus produced a garlic EO nano-emulsion with smaller and more homogeneous particles than those obtained with low-energy methods. Ricupero et al. (2022) developed a garlic EO nano-emulsion by using the self-emulsifying process coupled with sonication. Despite the ingredients were the same used in this study, the droplet size of that nano-emulsion was larger (176.23 ± 0.9 nm) and less homogeneous (PDI=0.18) than the one obtained during this research using a high-pressure microfluidizer. The stability over-time of an insecticide formulation is one of the key factors for a successful market and diffusion in real operating conditions. The proposed nano-emulsion demonstrated an excellent stability for at least three months and after one year, despite the increased size, the droplets remained in the nanometric range. Furthermore, the low PDI registered over time (from 0.05 to 0.17) revealed a better stability than other similar formulations; as an example, Long et al. (2020) produced several garlic EO nano-emulsions with relatively higher values of polydispersity index (PDI> 0.22).

The use of high relative amount of surfactant guarantees the production of very small droplets with an optimal size distribution (i.e., PDI tending towards zero). Nevertheless, the amount of surfactant should be reduced as much as possible when insecticides are developed for use on crop plants. Indeed, these substances at high concentrations can

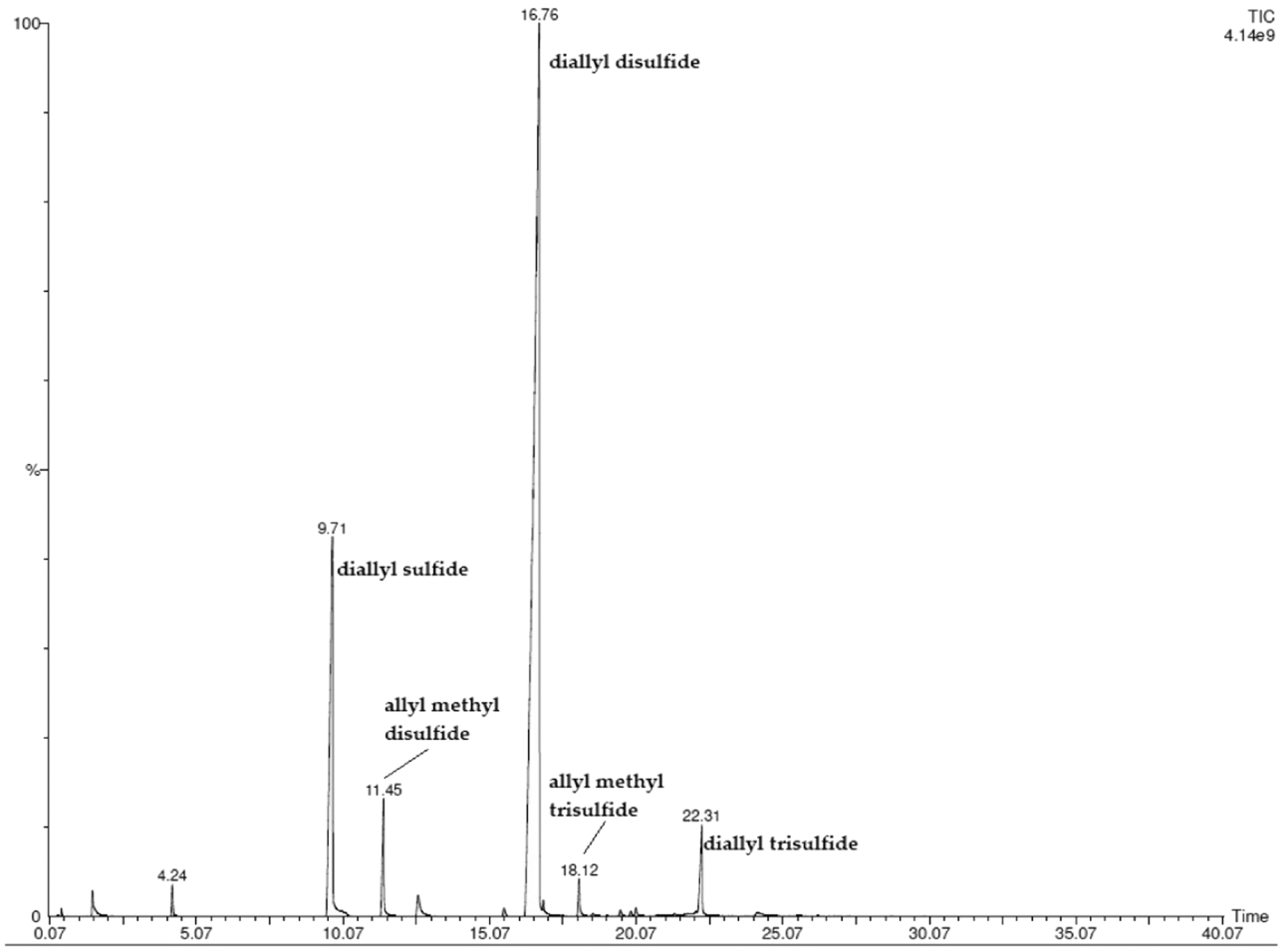

Fig. 1. GC-MS chromatogram of crude garlic EO.

negatively affect the plant growth and the permeability of the cell membranes, increase the absorption of contaminants and cause vegetable tissue damage (John et al., 1974; Knoche et al., 1992; Falk et al., 1994; HESS and FOY, 2000; Liu, 2004; Shin et al., 2021). The nano-emulsion produced during this study contained a lower amount of surfactant (EO:surfactant ratio = 3:1 w:w) compared to other studies. Mossa et al. (2018) developed several oil-in-water garlic EO-based nano-emulsions containing the EO and Tween 20 at different ratios, but always prevailing surfactant to EO (i.e., 1:1, 1:1.1, and 1:1.2 w/w). The same authors reported a stability over time and a particle dimension like our nano-emulsion. Similarly, Hassanzadeh et al. (2022)) developed different garlic EO-based nano-emulsions using different EO concentrations; keeping the oil/surfactant ratio constant (1:1), the particle dimensions and the PDI increased with the final concentration of both EO and Tween 80. An ideal insecticide formulation, apart the efficacy against the target pests, should have features that make it easy to handle in real operative condition. Among these characteristics a high a.i. concentration represents a key aspect. In this regard, many proposed EO-based nano-insecticides were characterised by a low a.i. concentration, which could cause economic and logistical disadvantages in real conditions (Duarte et al., 2015; Moghimi et al., 2016; Machado et al., 2023; Ntalli et al., 2023). The nano-emulsion prepared here, in contrast, contained 15% of garlic EO that, to the best of our knowledge, is one of the highest concentrations reached for this kind of formulation.

Insects belonging to Pseudococcidae family comprise several pests of agriculture and ornamental crops whose control is difficult because their small body and cryptic nature. The control of these pests is mainly carried out by the application of synthetic pesticides that, as well known, negatively impact on the environment. To overcome this criticisms, different EO-based insecticides, extracted from Lamiaceae, Rutaceae, Myrtaceae, Zingiberaceae and Euphorbiaceae, were tested against these pests (Avila et al., 2022). The application of garlic EO or its extract, formulated or not, as pesticides has gained increasing interest and many studies on garlic's pesticidal activity targeted several taxonomic groups such as: Lepidoptera (Ricupero et al., 2022; Ben Abdallah et al., 2023), Termites (Srivastava et al., 2021), Coleoptera (Yang et al., 2010; Plata-Rueda et al., 2017), Mallophaga (Abdel-Meguid et al., 2022), Mosquitoes (Thomas and Callaghan, 1999), Eriophyid (Mossa et al., 2018). On the other hand, the bioactivity of several EOs was tested towards different biological traits of *P. citri*. Among the most effective ones, fumigation with *Thymus capitatus* EO induced mortality in this pest with a LC_{50} value of 7.2 mg /L of air after 24 h, while *Mentha pulegium* EO revealed a LC_{50} value of 11.26 mg/L of air in contact toxicity trials (Attia et al., 2022; Alloui-Griza et al., 2022). However, the results of EO application depend on the botanical source. As an example, EOs from anise (*Pimpinella anisum*), rosemary (*Rosmarinus officinalis*), peppermint (*Mentha piperita*), Turkish oregano (*Origanum onites*) and thyme (*Thymus vulgaris*) showed a variable repellent activity depending on both the doses as well as the time (Erdemİr and Erler, 2018). In addition, some of the these EOs cause oviposition deterrence and egg-hatching inhibitory effects (Erdemİr and Erler, 2018).

Despite its potential as insecticide, the use of garlic EO against the citrus mealybug has been poorly investigated especially when referring to nano-formulations. The application of this EO, diluted in methanol, against the third instar nymphs of a close related species, the tea mealy bug *Pseudococcus viburni* (Hemiptera: Pseudococcidae), determined a LC_{50} values of 0.42% and 0.31% after 24 and 48 h (Ramzi et al., 2022). Spray treatments of garlic EO against *Paracoccus marginatus* Williams

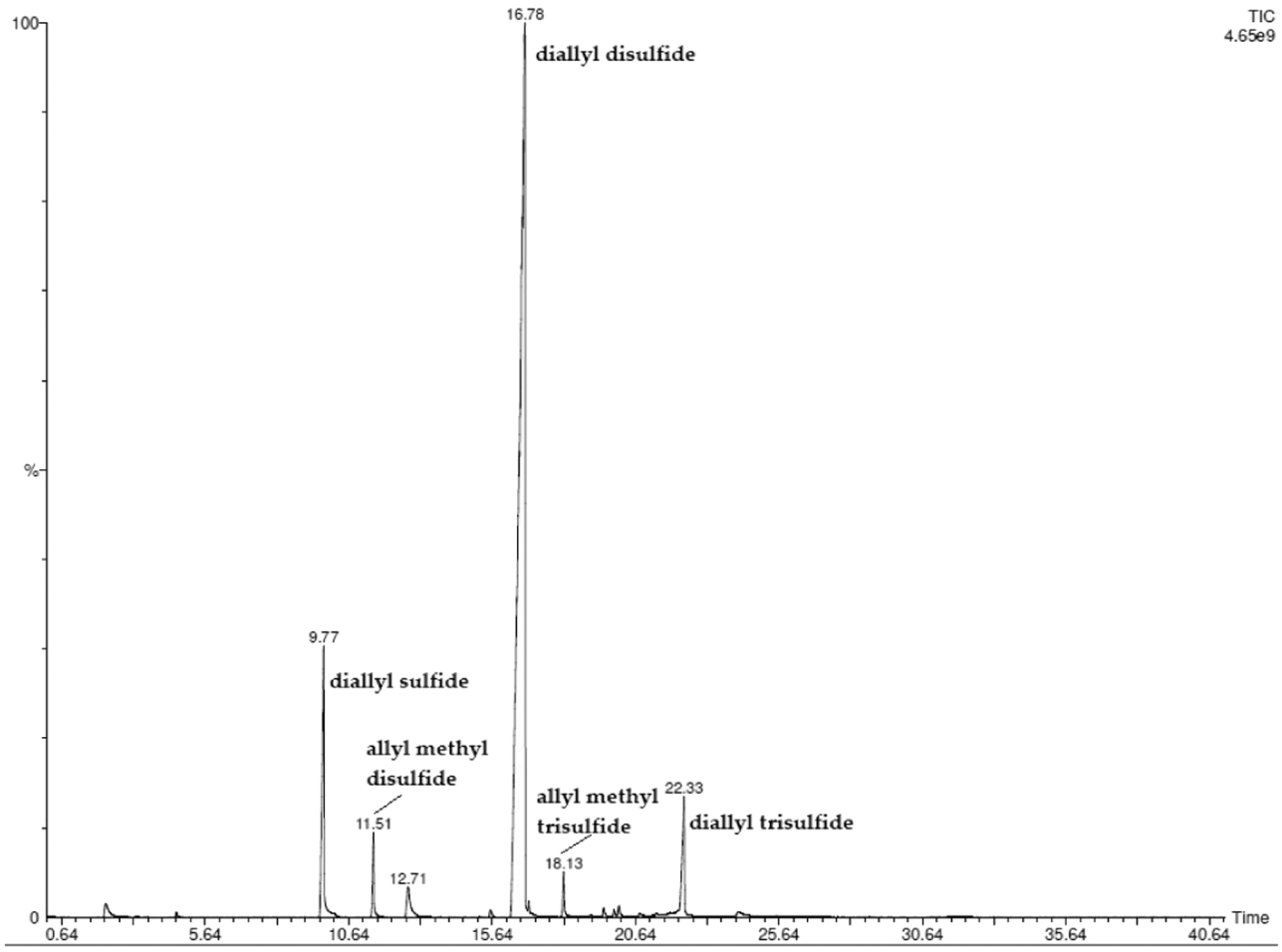

Fig. 2. GC-MS chromatogram of garlic EO in nano-emulsion.

Table 2
Physical characteristics of garlic essential oil nano-formulation during storage. Values are means (± standard deviation) of three replicates. Different letters indicate statistical differences among the values registered at the different storage times ($p < 0.05$).

Time	Size (nm)	PDI[a]	Zeta Potential (mV)
24 h	64.5 ± 0.51a	0.05 ± 0.01a	-17.6 ± 1.25a
3 months	82.8 ± 0.45b	0.12 ± 0.02b	-20.83 ± 0.23b
1 year	221.4 ± 2.67c	0.17 ± 0.02c	-30.4 ± 0.52c

[a] PDI = Polydispersity index

and Granara de Willink (Hemiptera: Pseudococcidae) showed a dose dependent mortality trend influenced by time (24–72 h after treatment), as well as by the use of adjuvants (i.e., isopropyl alcohol or paraffin oil) (Mwanauta et al., 2023).

One of the most interesting aspect related to the use of EOs against pests is their assumed selectivity towards non-target organisms (e.g., natural enemies and pollinators) that is considered a misperception to a some extent (Haddi et al., 2020). In this study, the nano-formulation was safe toward *C. moutrouzieri* adults. However, EO-based insecticides could have various negative impacts on biological control agents since the supposed safety against non-targets is related to the few studies available from the literature. The negative effects towards non target organisms depend on different variables (i.e. EO, application rate, route of exposure, non-target insects) and can include mortality, decreased respiration rate, diminished predatory capacity, and lower rates of parasitization, among other detrimental effects (Giunti et al., 2022a). *Mentha pulegium* EO displayed acute toxicity against three citrus scale

Table 3
Acute (direct) and residual contact (indirect) toxicity of garlic essential oil against the second and third instars of *P. citri* 24 and 48 h after the exposure. Values were considered significantly different if their 95% fiducial limits did not over-lap.

[a]LC	Method	Time	% garlic EO (95% CI[b])	Chi square (df[c])	*p*
50	Direct	24 h	0.363 (0.236–0.489) a	6.642(3)	0.249
		48 h	0.248 (0.166–0.364) a	5.805(3)	0.326
	Indirect	24 h	0.852 (0.783–0.939) b	1.758(3)	0.624
		48 h	0.782 (0.713–0.859) b	0.993(3)	0.803
90	Direct	24 h	1.182 (0.820–2.135) A	6.642(3)	0.249
		48 h	0.967 (0.647–1.903) A	5.805(3)	0.326
	Indirect	24 h	1.154 (1.024–1.472) A	1.758(3)	0.624
		48 h	1.088 (0.965–1.374) A	0.993(3)	0.803

[a] LC= Lethal concentration; CI
[b] = Confidence interval; df
[c] = degrees of freedom. Different letters indicate statistical differences between treatments within the same LC value.

insect species (*P. citri*, *A. aurantii*, and *C. aonidum*), while it did not induce mortality towards *C. montrouzieri* adults. (Attia et al., 2022). A commercial EO-based formulation (Prev-am®), based on D-limonene (the main compound of sweet orange EO), exhibited high mortality against *Dactylopius opuntiae* (Cockerell) (Hemiptera: Dactylopiidae), while exerting minimal influence on both adults and larvae of *C. montrouzieri* (El Aalaoui et al., 2019). Conversely, *Thymus capitatus* EO was significantly harmful towards *C. montrouzieri* adults at 10 and 20 µl/L air resulting, after 3 days, in a mortality rate exceeding 90%

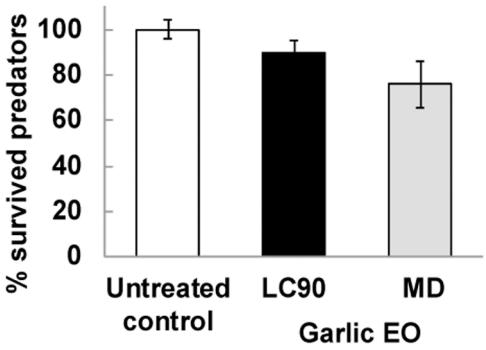

Fig. 3. Mean (±) survival values for *Cryptolaemus montrouzieri* specimens after topical contact exposure to garlic essential oil at two lethal concentrations applied for the target pest *Planococcus citri* (LC_{90} and Maximum Dose) and distilled water (untreated control). No statistical differences ($p > 0.05$) were highlighted among the different treatments (GLM – ANOVA, Duncan's post-hoc test).

among the exposed beetles (Alloui-Griza et al., 2022). The predation ability could be also influenced by the treatments carried out for pest control. Bibi et al. (2022) observed that *C. montrouzieri* adults consumed more preys when they were treated with citrus oil rather than with an organophosphate insecticide (profenofos). Furthermore, the mortality of *C. montrouzieri* adults was notably higher when consuming citrus mealybugs treated with profenofos, in comparison to those treated with citrus oil (Bibi et al., 2022). Summarizing, the majority of studies dealing with side-effects caused by EOs against *C. montrouzieri* reported none or limited impact of botanicals (Giunti et al., 2022a). Similarly, the side effects of a garlic-EO based nano-emulsion were tested against *Nesidiocoris tenuis* (Reuter) (Hemiptera: Miridae) adults proving to be harmless, although the exposure to this bioinsecticide affected the progeny produced by this mirid predator (Ricupero et al., 2022).

The research and development of insecticides derived from EOs has seen a significant increase in the last decades. Despite extensive research demonstrating their potential, the commercialization of these bio-insecticides at a large scale is at an embryonic stage. The reasons for their low uptake lie in some constraints, such as the unclear regulatory approval processes and the cost of some raw materials which are very different among Countries (Giunti et al., 2023). Nevertheless, the future for biopesticides based on EOs seems promising thanks to the growing global demand for sustainably produced and/or organic food.

5. Conclusions

The high-pressure micro-fluidization technique has enabled the production of a high concentrated (15%) garlic EO nano-emulsion by using low amount of surfactant that make this biopesticide suitable for the application in real operative conditions. The obtained optimal physical properties, allowing a good stability over time, is another positive result. The efficacy of the developed formulation against *P. citri* and the absence of toxic effects towards the coccinellid predator *C. montrouzieri* suggest that the application of garlic EO could be a viable option for managing *P. citri* populations in citrus orchards even in the presence of the adult predators. However, lethal effects of the tested EO on predatory larvae and their sublethal effects on either *C. montrouzieri* larvae or adults should also be assessed in future studies for a more complete toxicity profile of the developed formulation.

CRediT authorship contribution statement

MR, LZ, SG, VP, and OC conceived the research. AM, IL, GM, SG performed the research. OC conceived and prepared the nano-formulations. AM, GG and OC analyzed and interpreted the data. AM wrote the first draft of the manuscript. All authors commented on the manuscript. SG, LZ, VP, OC provided the materials and access to the laboratories. All authors read, revised, and approved the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.

Acknowledgment

This study was carried out within the project ASTER from Call Prima Section 2 2021—Multi-Topic, the Agritech National Research Center and received funding from the European Union Next-Generation EU (PIANO NAZIONALE DI RIPRESA E RESILIENZA (PNRR) – MISSIONE 4 COMPONENTE 2, INVESTIMENTO 1.4 – D.D. 1032 17/06/2022, CN00000022). This manuscript reflects only the authors' views and opinions, neither the European Union nor the European Commission can be considered responsible for them.

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CHAPTER 6

Green pest control strategies: Essential oil-based nano-emulsions for *Delottococcus aberiae* De Lotto (Hemiptera: Pseudococcidae) management

Green pest control strategies: essential oil-based nano-emulsions for *Delottococcus aberiae* DeLotto (Hemiptera: Pseudococcidae) management

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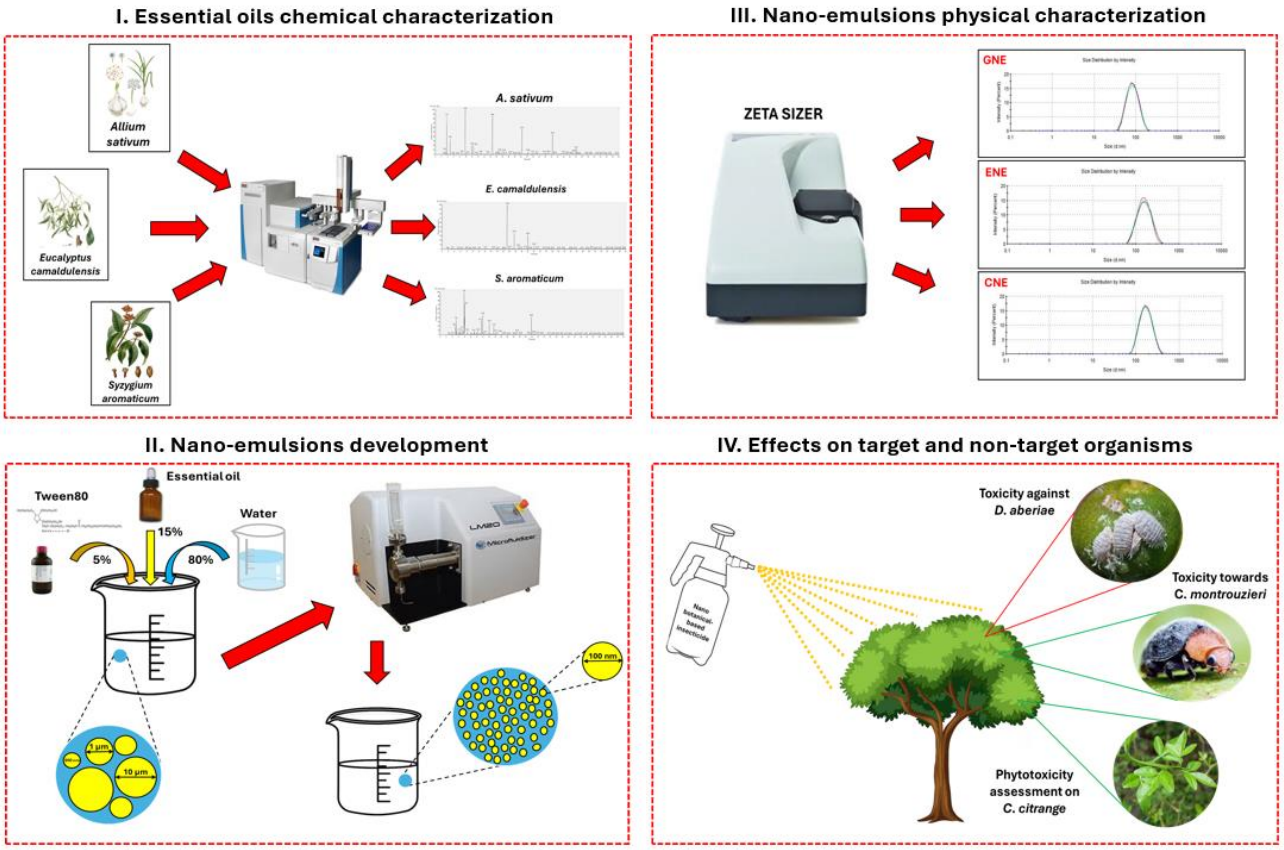
ABSTRACT

Increasing restrictions on synthetic pesticides due to environmental and health concerns have driven the search for alternative, environmentally friendly pest management strategies. Essential oils (EOs) from plants like garlic (*Allium sativum*), clove (*Syzygium aromaticum*), and eucalyptus (*Eucalyptus camaldulensis*) have shown promise as bioinsecticides. However, their volatility, low water solubility, and short persistence limit their practical application in Integrated Pest Management (IPM) programs. To address these challenges, we developed nano-emulsions of these EOs using a high-pressure microfluidisation technique, achieving stable formulations with nano-sized droplets (<200 nm) and optimal polydispersity index (PDI) and zeta potential values. The insecticidal efficacy of these EO-based nano-emulsions was tested against the invasive citrus pest *Delottococcus aberiae*, with garlic nano-emulsion (GNE) exhibiting the highest mortality (100% within 24 hours), significantly outperforming clove and eucalyptus formulations. GNE exhibited a dose-response mortality against *D. aberiae* while demonstrating high safety (100% of survival) towards *Cryptolaemus montrouzieri* and no phytotoxicity on citrus plants. Moreover, gene expression analysis revealed that GNE application triggered the overexpression of key genes involved in plant defense pathways, including *ICS2*, *NPR1*, *PAL*, and *MYC2*, suggesting the activation of both salicylic acid (SA) and jasmonic acid (JA) signaling pathways. This dual action—

direct pest control and enhancement of plant defenses—positions GNE as a powerful tool in sustainable citrus pest management, with potential applications in real-world pest control. The study underscores the potential of EO-based nano-emulsions as a safe, effective, and environmentally sound alternative to chemical insecticides.

KEYWORDS: citrus pest, garlic, clove, eucalyptus, non-target organisms, plant defenses, sustainable agriculture

GRAPHICAL ABSTRACT:



HIGHLIGHTS

- Developed essential oil-based nano-emulsions showed long-term stability
- Garlic EO-based nano-emulsion achieved the highest mortality rates for *D. aberiae*
- No phytotoxicity or adverse effects on non-target organisms were observed
- Garlic EO-based nano-emulsion enhanced natural plant defenses

1. INTRODUCTION

The mealybug *Delottococcus aberiae* (De Lotto) (Hemiptera: Pseudococcidae) is an invasive pest native originating from South Africa that has recently spread to European citrus orchards (Beltrà et al., 2012; Pérez-Rodríguez et al., 2018). It has been reported to feed on several tropical and subtropical plants including citrus, coffee, guava, pear, and olive (De Lotto 1961; Miller DR and Giliomee JH 2011). This mealybug causes severe damage to citrus fruits, leading to deformities and reduced fruit size. Furthermore, the excreted honeydew promotes the growth of sooty mould fungi, which hinders photosynthesis. These negative effects, combined with the absence of *D. aberiae* effective natural enemies, have resulted in significant economic and environmental consequences (Martínez-Blay et al., 2018; Tena et al., 2017). Among the natural enemies, *Cryptolaemus montrouzieri* (Mulsant) (Coleoptera: Coccinellidae), a well-known predator of mealybugs in citrus orchards, contributes to the control of *D. aberiae* (Pérez-Rodríguez et al., 2016). However, on its own, it is not sufficient to fully manage the pest. Consequently, since the *D. aberiae* introduction in Europe, control strategies have predominantly relied on synthetic pesticides (Franco et al., 2009; Pérez-Rodríguez et al., 2017; Tena et al., 2017; Vacas et al., 2019). The extensive use of conventional pesticides has led to adverse effects, including environmental pollution and potential health risks to humans (Mata et al., 2024; Saroop and Tamchos, 2024). In response to these negative consequences, the efficacy of natural substances, in particular essential oils (EOs), as insecticides has been extensively studied over the last decade (Assadpour et al., 2024; Pavela and Benelli, 2016; Said- et al., 2017). EOs are complex phyto-complexes containing a wide range of substances with properties that make them suitable as bioinsecticides, such as proven toxicity against several pests, wide availability and distribution, and low toxicity towards non-target organisms such as natural enemies, pollinators, or mammals (Giunti et al., 2022).

The biological activity of EOs from different botanic families (e.g., Lamiaceae, Myrtaceae, Amaryllidaceae, Rutaceae, Apiaceae) has been investigated against other mealybugs (Avila et al., 2022). Mostafa, et al. (2018) demonstraed the efficacy of different EOs against *Phenacoccus solenopsis* (Tinsley) (Hemiptera: Pseudococcidae), noting that *Thymus vulgaris* EO exhibited good insecticidal activity with LC_{50} values of 29.03 and 15.04 ppm 24 and 72 hours post-exposure, respectively. Similarly, other studies have highlighted the insecticidal effectiveness of EOs from *Salvia rosmarinus, Cymbopogon citratus, Eucalyptus melliodora,* and *Mentha spp.* against the same pest (Roozdar et al. 2020; Saad et al. 2021). Futhermore, other studies have reported the efficacy of these EOs against *Planococcus ficus* (Signoret), *Planococcus citri* (Risso), *Ferrisia virgata*

(Cockerell) and *Nipaecoccus nipae* (Maskell) (Hemiptera: Pseudococcidae) (Elhefny et al., 2023; Erdemir and Erler, 2017; Hassan et al., 2023; Karamaouna et al., 2013; Peschiutta et al., 2017).

Despite the biological efficacy of the EOs as insecticides, certain limitations, such as volatility, photo-lability, poor water solubility, or flammability, pose challenges for their practical application. In recent years, nanotechnology has shown promise in overcoming these limitations, providing new, environmentally friendly tools based on EOs (Giunti et al., 2023). In Integrated Pest Management (IPM), nanotechnology typically involves the encapsulation of active ingredients within nano-delivery systems (e.g., nano-emulsions and nanoparticles) that exhibit nanoscale physical properties (Bakkali et al., 2008; Burt, 2004; Campolo, 2022). Research has demonstrated that nano-insecticides based on EO formulations not only improve the biological efficacy of EOs but also enhance their stability over time, outperforming raw or unformulated EO (Campolo et al., 2020a; Modafferi et al., 2024a).

Recently, it has been demonstrated that the application of essential oils (EO), due to their high content of secondary metabolites, can activate the defenses of the plants to which they are applied. In this context, EO treatments increased the expression of genes related to jasmonic acid (JA) and salicylic acid (SA) signaling pathways, enhancing the plants' resistance to pests and pathogens in tomatoes and peppers (Ben Abdallah et al., 2023; Ricupero et al., 2023). However, whether this defense activation could be achieved with EO-based nano-emulsions in a perennial crop like citrus still needs to be discovered.

This study aimed to develop stable and effective EO-based nano-emulsions with a high EO: surfactant ratio (3:1) through the high-pressure microfluidization technique. Firstly, the EOs (i.e. garlic, clove and eucalyptus) and their nano-emulsions were chemically and physically characterized through gas chromatography-mass spectrometry (GC-MS) and dynamic light scattering (DLS) analyses, respectively. Then the EO-based nano-emulsions were used to investigate the biological activity against the 2nd instars of *D. aberiae* and the most efficacy nano-emulsion (i.e. garlic nano-emulsion) was subjected to further investigations in order to assess its toxicity on non-target organisms such as *C. montrouzieri* and citrus plants. At the same time, the effects of garlic nano-emulsion on transcriptional levels of different genes involved in plant defense mechanisms were also investigated.

2. MATERIALS AND METHODS

2.1 Plant material

Carrizo citrange (*Citrus sinensis* × *Poncirus trifoliata*) plants used in the experiments were obtained from seeds and cultivated in a growth chamber of the Instituto Valenciano de Investigaciones Agrarias (IVIA) (Valencia, Spain) at 25 ± 1°C, relative humidity of 60%, and a photoperiod of 16:8 h light:dark (L:D). Plants were used when they reached 3 months old and had 8 to 9 fully expanded leaves.

2.2 Insect rearing

Delottococcus aberiae was reared for several generations at the facilities of the IVIA on lemon fruits [*Citrus* × *limon* (L.) Osbeck (Sapindales: Rutaceae) in a dark climate chamber under constant climatic conditions (25 ± 1°C and 60 ± 5% relative humidity RH). *Cryptolaemus montrouzieri* adults were purchased from Bioline AgroSciences Iberia (Almeria, Spain).

2.3 Chemical compositions of EOs

Commercial *Allium sativum* EO (GEO) and *Syzygium aromaticum* EO (CEO) were purchased from Esperis S.p.A. (Milan, Italy). *Eucalyptus camaldulensis* EO (EEO) was steam extracted from leaves collected from plants growing in southern Italy (Reggio Calabria, Italy).

The chemical characterization of the EOs was carried out following the methods described by Giunti et al. (2019). Briefly, a Thermo Fisher TRACE 1300 GC with a MEGA-5 capillary column (30 m × 0.25 mm; coating thickness = 0.25 µm) and a Thermo Fisher ISQ LT mass detector (ionization mode: EI; scan time: 1.00 s; scan mass range: 30–300 m/z) were used setting injector and transfer line at 250 and 240 °C, respectively, and a temperature ramp from 60 to 240 °C at 3 °C min^{-1} (carrier gas: He 1 mL min^{-1}). The pure EO was diluted (1:10 v:v) in hexane (95%, Sigma Aldrich, Munich, Germany), and 0.2 µL were injected at a split ratio of 1:30. The identification of peaks was made using computer matching against the commercial libraries (NIST 05, Wiley FdaviesFNSC and ADAMS) comparing linear retention indices (LRI). The LRIs were calculated using the formula of Van den Dool & Kratz (1963) by comparing the retention times of the compounds to be identified with those of a standard mixture of alkanes (C8-C20 saturated alkanes standard mixture, Supelco®, Bellefonte, PA, USA) which was analyzed in GC-MS under the identical operating conditions as the sample (van Den Dool and Dec. Kratz 1963; Masada 1976; McLafferty and Stauffer 1989; Davies 1990; Jennings 2012; Adams et al. 2017).

2.4 Formulation and physical characterization of nano-emulsions

Allium sativum nano-emulsion (GNE), *E. camaldulensis* nano-emulsion (ENE), and *S. aromaticum* nano-emulsion (CNE) were obtained using a high-pressure micro-fluidization (HPM) technique, following the methods described by Modafferi et al. (2024c). Firstly, EO and Tween 80® (Polyoxyethylene (20) sorbitan monooleate, Sigma Aldrich, Munich, Germany) (ratio 3:1 w: w) were mixed using a magnetic stirrer (5 min at 6000 RPM) to obtain a homogeneous organic phase. Then, the raw emulsion was prepared by slowly adding (1 mL/min) double distilled water to the organic phase (ratio 4:1 w:w). The resulting raw emulsion (EO 15%, Tween 80 5%, and water 80% w: w) was mixed for 5 min at 7000 rpm. The final mixture was homogenized five times at 30,000 PSI through an HPM apparatus (LM20 Microfluidizer™ Processor, USA) to obtain a homogeneous nano-emulsion. The interaction chamber was immersed in an ice bath to maintain a low temperature (<10°C) during the HPM process. The resulting nano-emulsion was then stored in aluminum bottles and kept at 4°C. This process was repeated for each EO.

The developed EO-based nano-emulsions were then physically characterized to assess the droplet dimension (Z-average size), polydispersity index (PDI), droplet surface charge (ζ-potential) and stability over time (1 day, 1 month, and 1 year). The analyses used a Dynamic Light Scattering (DLS) apparatus (Zetasizer Nano, Malvern®). The measurements were conducted by diluting the developed nano-emulsions in double distilled water (ratio 1:200 v:v). To assess the droplet size and droplet surface charges, 1mL and 0.75mL of diluted formulations were used, respectively.

2.5 Insecticidal activity against target insect

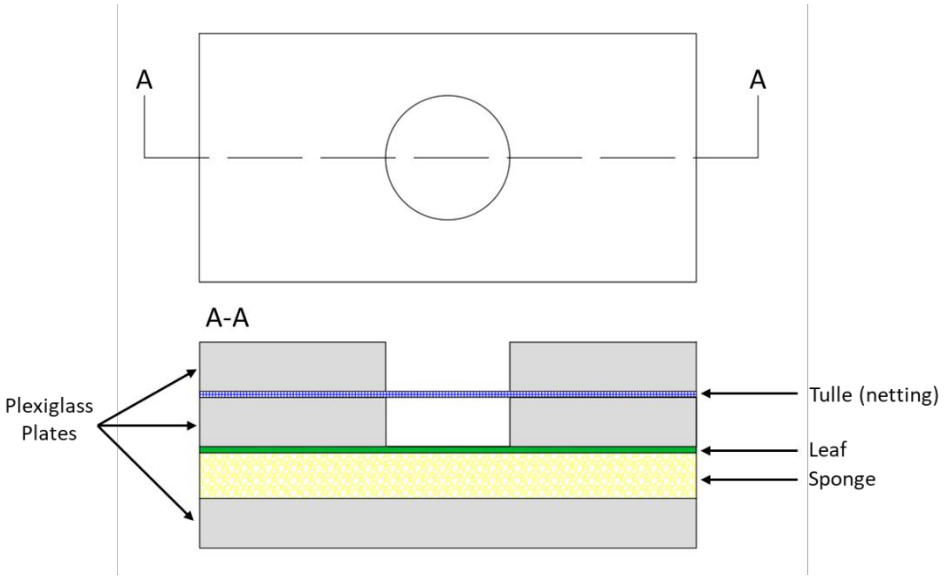
The insecticidal activity of the developed nano-emulsions was evaluated through residual contact toxicity trials using the leaf-dip method (Modafferi et al., 2024c). To assess the efficacy of each nano-formulation and to discard that they did not have the potential to be used in real field conditions, preliminary trials were carried out at a high amount of EO (2.5%). Bioassays were carried out by individually immerging rectangular bean pod sections (2 x 4 cm) for 10 seconds in the emulsion (2.5% of EO). After that, the treated sections were dried at room temperature and then placed in ventilated plastic Petri dishes (Ø 5.5 cm). Subsequently, 15 *D. aberiae* second instar individuals were gently placed on the treated bean pod surface. The trials were conducted under laboratory conditions, and the experimental arenas were maintained under the same rearing conditions as the abovementioned ones. Insects were considered dead if immobile after stimulation with a fine brush. Mortality data was recorded 24, 48, and 72 hours after the exposure.

The preliminary results showed that only GNE provided a good mortality efficacy, so only this oil was used for further bioassays (see result section 3.2). The efficacy of serial dilutions of GNE (0.46, 0.625, 0.93, 1.25, and 1.87% w:w of EO), was evaluated against *D. aberiae* as described above. Each dilution was replicated eight times, and water and deltamethrin (Decis, Bayer Crop Science, San Joan Despí, Barcelona, SP) were used as negative and positive control treatments, respectively.

2.6 Side-effects on *Cryptolaemus montrouzieri*

Toxicity on *C. montrouzieri* adults was assessed through a residual contact toxicity trial using the LD_{50} (1.14% of EO) and LD_{90} (2.20% of EO) estimated 72 hours after the exposure against *D. aberiae* (see Table 2). The experiment was replicated ten times for each LD value, negative control (water), and positive control (deltamethrin, Decis, Bayer Crop Science, San Joan Despí, Barcelona, SP). The positive control was tested at the maximum label concentration (15 ml/hl). The bioassays were conducted on non-infested, fully expanded detached leaves from *C. citrange* seedlings. The experimental unit was a modified Huffaker cell (Fig. 1) (Abad-Moyano et al., 2009).

Figure 1. Schematic representation of the modified Huffaker cell used in the residual contact toxicity with *Cryptolaemus montrouzieri*.



This unit consisted of a bottom PVC plate (80 x 40 x 5 mm) and two top PVC plates (80 x 40 x 10 mm). The top plates had a central circular hole (Ø 2 cm). On each bottom plate, a *C. citrange* leaf treated with the corresponding treatment was placed on a wet cleaning cloth (80 x 40 mm) (Vileda®, Freudenberg Home and Cleaning Solutions GmbH, Weinheim, Germany), ensuring that

the leaf substrate formed the bottom of the arena. A muslin cloth was placed between the two bottom plates to allow ventilation in the arena. Then, 5 adults were placed on the citrus leaf surface, and the arena was assembled by covering the bottom plate with both top plates. The three plates were secured together with two rubber bands (Supplementary Figure 1 SF.1). The arenas were kept in a climate chamber at 25 ± 2°C, 65 ± 10% R.H., and a photoperiod of 14:10 h (L:D) throughout the experiment. Mortality was recorded 24, 48, and 72 hours after the exposure. Insects were considered dead if they were not able to walk. Eggs of *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae) were offered daily as food to the predators until the end of the experiment.

2.7 Phytotoxicity on citrus seedlings

The phytotoxic effects of GNE were observed on *C. citrange* plants. Three treatments were carried out using the estimated LD_{50}, LD_{90} (1.14 and 2.20% of EO, respectively) (see Table 2), and negative control (water). Each treatment was replicated six times. Citrus seedlings with an average height of 25 cm and approximately the same number of leaves were used. The plants were individually treated with a hand sprayer until runoff, placed in different climate chambers (one per treatment),

$$Pi = \sum_{j=0}^{n} \left(\frac{DLj}{TL} \times \frac{DC}{n-1}\right)$$

and maintained under the same conditions (25 ± 2°C, 65 ± 5% R.H. and a photoperiod of 14:10 h L:D). The phytotoxic effects of GNE were checked 24 hours, 3, 7, and 14 days after treatment, recording the number of damaged leaves and the severity of the damage. The severity of damage was classified following the method described by Campolo et al. (2017):

Where *DL* is the number of damaged leaves for each damage severity class *j*; *TL* is the total number of leaves sprayed; *DC* is the damage severity class (0= no damage; 1=leaf surface with only chlorosis; 2=leaves with evident necroses and 3=dead leaves; *n* is the number of damage severity classes. The *Pi* ranges from 0 (no damage) to 1 (dead leaves).

2.8 Plant defenses on citrus seedlings

The ability of GNE to induce the expression of genes involved in plant defenses was evaluated at the same concentrations used in the phytotoxicity assessment (see section 2.5.2). Five genes were analyzed via Real-time PCR (rt-PCR). Specifically, the expression levels of *ICS2* (Isochorismate Synthase 2, Chloroplastic), *NPR1* (Non-Pathogenesis Related Protein 1) and *PAL* (phenylalanine ammonia-lyase), associated with the Salicylic acid (SA) pathway, as well *JAR1* (jasmonate resistant

1) and *MYC2* (Transcription factor MYC2, DNA binding protein), which are marker for Jasmonic acid (JA) pathway, were investigated (Alfarez et al. 2018; Pérez-Hedo et al. 2024). *GADPH* (Glyceraldehyde-3-phosphate dehydrogenase) was used as an internal control for normalization. The nucleotide sequences of the gene-specific primers are provided in Supplementary Table 1 (ST1).

The experiment included seven replicates for each LC value and control (water) treatment. Citrus seedlings were treated under the same conditions as those used in phytotoxicity trials (see section 2.5.2). Twenty-four hours post-treatment, the apical region of each plant was excised, wrapped in foil paper, and immediately ground in liquid nitrogen. Samples were stored at -80°C until the RNA extraction. Following the manufacturer's recommendations, total RNA was extracted using NZYol (NZYTech, Lisboa, Portugal). Then, 10 µg of total RNA was treated with a TURBOTM DNA-free kit (Ambion, Life Technologies, CA, United States) to remove the DNA contamination. Reverse transcription was performed using PrimeScript RT reagent kit (TAKARA BIO INC., Japan) starting from 1 µg of RNA-DNA free template. Real-time PCR amplification was performed in LightCycler 480 System (Roche Molecular Systems, Inc., Switzerland), using 5 µl of NZYSupreme qPCR Green Master Mix (2x) (NZYTech, Lisboa, Portugal), 0.5 µl of forward and reverse primers (10 mM) and 2µl of cDNA, resulting in a total reaction volume of 10 µl. The reaction was performed using the following PCR conditions: 1 cycle at 95°C for 2 minutes, 40 cycles at 95°C for 15 seconds, 58°C for 30 seconds, 72°C for 30 seconds, and a final melting cycle at 95°C for 5 seconds and 65°C for 1 minute. The relative fold gene expression of the samples was calculated using the 2 ΔΔCT method described by Ricupero et al. (2023).

2.9 Statistical analysis

Changes in the physical characteristics of the EO-based nano-emulsions (GNE, ENE, and CNE) over time were analyzed using analysis of variance (ANOVA) with particle size, polydispersity index (PDI), and zeta potential as dependent variables, and the nano-emulsions used as fixed factors. All data met the assumptions required for parametric testing, including normality and homoscedasticity of variance ($P > 0.05$). Mortality data for *D. aberiae* were corrected for control mortality using Abbott's formula (Abbott, 1925). Differences in exposure times in the preliminary test were also subjected to ANOVA analysis. The concentration-mortality response was evaluated by Probit analysis, in which the LD_{50} and LD_{90} values and their fiducial limits were estimated using the results obtained 24, 48, and 72 hours after the treatment with GNE. LD values were considered significantly different if their 95% fiducial limits did not overlap. The effects on *C. montrouzieri* adult

mortality following treatment with LD_{50} and LD_{90} were assessed with the Kruskal-Wallis test. The ability of GNE dilutions to enhance plant defenses on *C. citrange* plants was analyzed using ANOVA, and multiple comparisons were conducted using Tukey's HSD post hoc test. All statistical analyses were performed using IBM® SPSS® StatistICS2 v. 23 (IBM Corp. Released 2015. Armonk, NY, USA).

3. RESULTS

3.1 Characterisation of EOs and EO-based nano-emulsions

The GC-MS analysis identified twenty-three, twenty-six, and sixty-three components, accounting for 97.87%, 99.45%, and 95.33% of the total area for GEO, CEO, and EEO, respectively (SF.2). GEO consisted primarily of sulfur compounds (97.87%) (ST.2). At the same time, CEO was composed of aldehydes (0.03%), monoterpenes (0.05%), oxygenated monoterpenes (0.06%), phenylpropenes (88.65%), esters (0.16%), sesquiterpenes (8.69%) and oxygenated sesquiterpenes (1.83%) (ST.3). In contrast, EEO included aldehydes (0.01%), monoterpenes (61.03%), oxygenated monoterpenes (17.56%), phenylpropenes (6.76%), esters (0.01%), sesquiterpenes (1.40%) and oxygenated sesquiterpenes (8.56%) (ST.4).

The physical characteristics of the EO-based nano-emulsions developed are presented in Table 1. All formulations demonstrated good stability over time, with particle size in the nanometric range, as confirmed by their negative zeta potential and low PDI values. The physical properties of the nano-emulsions varied depending on the type of EO used in each formulation. Specifically, the ENE displayed the smallest droplet size (62.5 ± 1.1 nm) one-day post-development ($F = 4399.1$; df = 2; $P < 0.01$), whereas GNE showed the smallest droplet size at both one month (96.4 ± 1.8 nm; $F = 3681.9$; df = 2; $P < 0.01$) and one year (215.7 ± 2.4 nm; $F = 513.1$; df = 2; $P < 0.01$) after the formulation. In contrast, the CNE exhibited the largest droplet size throughout the experiment, with particle size values ranging from 161.3 ± 0.7 nm on day one to 268 ± 2.5 nm after one year. A high-energy system allowed to develop nano-emulsions with low PDI values (< 0.25). In detail, one day after development, no statistical differences were observed between GNE and ENE ($F = 12$; df = 2; $P < 0.01$) with very low PDI values close to zero (0.07 ± 0.01 and 0.09 ± 0.03 for GNE and ENE respectively) after 1-month statistical differences were observed among all the developed nano-emulsion ($F = 105.6$; df = 2; $P < 0.01$), while 1 year after development, only GNE highlighted statistical differences ($F = 24.4$; df = 2; $P < 0.01$) with PDI values less than 0.2. Furthermore, considering the surface charge ($P < 0.05$), the EO plant source resulted in significant differences. Particularly, CNE registered the lowest negative surface charge at all observed times with values ranging from -24.7 ± 2.3 mV to -35.9 ± 0.4 mV 1 day ($F = 79.7$; df = 2; $P < 0.01$) and 1 year ($F = 598.5$; df = 2; $P < 0.01$), respectively.

Table 1. Physical characteristics (particle size, PDI, and zeta potential) of different developed EO-based nano-emulsions during storage.

TIME	NE[1]	Size (nm)	PDI[2]	Zeta Potential (mV)
1 day	GNE[3]	69.3 ± 2.1 a	0.07 ± 0.01 a	-15.3 ± 0.4 a
	ENE[4]	62.5 ± 1.1 b	0.09 ± 0.03 a	-11 ± 0.5 b
	CNE[5]	161.3 ± 0.7 c	0.15 ± 0.01 b	-24.7 ± 2.3 c
1 month	GNE	96.4 ± 1.8 a	0.09 ± 0.01 a	-22.8 ± 1.6 a
	ENE	101.3 ± 1.7 b	0.18 ± 0.01 b	-15.7 ± 0.4 b
	CNE	193.2 ± 0.9 c	0.14 ± 0.01 c	-27.1 ± 0.2 c
1 year	GNE	215.7 ± 2.4 a	0.16 ± 0.02 a	-30.6 ± 0.6 a
	ENE	230.5 ± 0.7 b	0.21 ± 0.01 b	-21.8 ± 0.4 b
	CNE	268 ± 2.5 c	0.21 ± 0.01 b	-35.9 ± 0.4 c

[1]Nano-emulsion; [2]Polydispersity index; [3]*Allium sativum* EO-based nano-emulsion; [4]*Eucalyptus camaldulensis* EO-based nano-emulsion; [5] *Syzygium aromaticum* EO-based nano-emulsion. Values represent the mean (± SD standard deviation) of three replicates. Different letters indicate statistical differences among nano-emulsions at the same storage time ($P < 0.05$).

3.2 Toxicity of EO-base nano-emulsions against *Delottococcus aberiae*

At the maximum application rates (2.5% EO), the different formulations displayed variable toxicity against second instars of *D. aberiae*, depending solely on the EO used (Fig. 2). In the water control treatments, no mortality was observed, whereas all specimens exposed to deltamethrin at the labeled dose (positive control) experienced 100% mortality. GNE exhibited very high toxicity against the mealybugs, achieving 100% mortality within 24 hours post-exposure. In contrast, the maximum mortality caused by CNE and ENE increased gradually over time. Specifically, CNE showed low mortality 24 and 48 hours after exposure (8 ± 7% and 23 ± 9%, respectively), which rose to 63 ± 5% at 72 h post-exposure. Conversely, ENE showed very low efficacy, with mortality rates reaching approximately 25 ± 4% at 72 hours post-exposure. Given the high efficacy of GNE, this nano-formulation was the only one further tested against the pest. Mortality data recorded at all observed times for the selected EO-based nano-emulsion (i.e., GNE) showed a dose-response relationship and fit well with the probit model ($P > 0.05$) (Table 2). However, no significant differences were observed across times within the same LD, as indicated by the overlapping fiducial limits.

Figure 2. Mortality % (mean ± SE) at 24, 48, and 72 hours after exposure to different developed EO-based nano-emulsions against *D. aberiae* in the preliminary trials (2.5% of EO). Different letters indicate statistical differences among exposure times within the same EO-based nano-emulsion ($P < 0.05$).

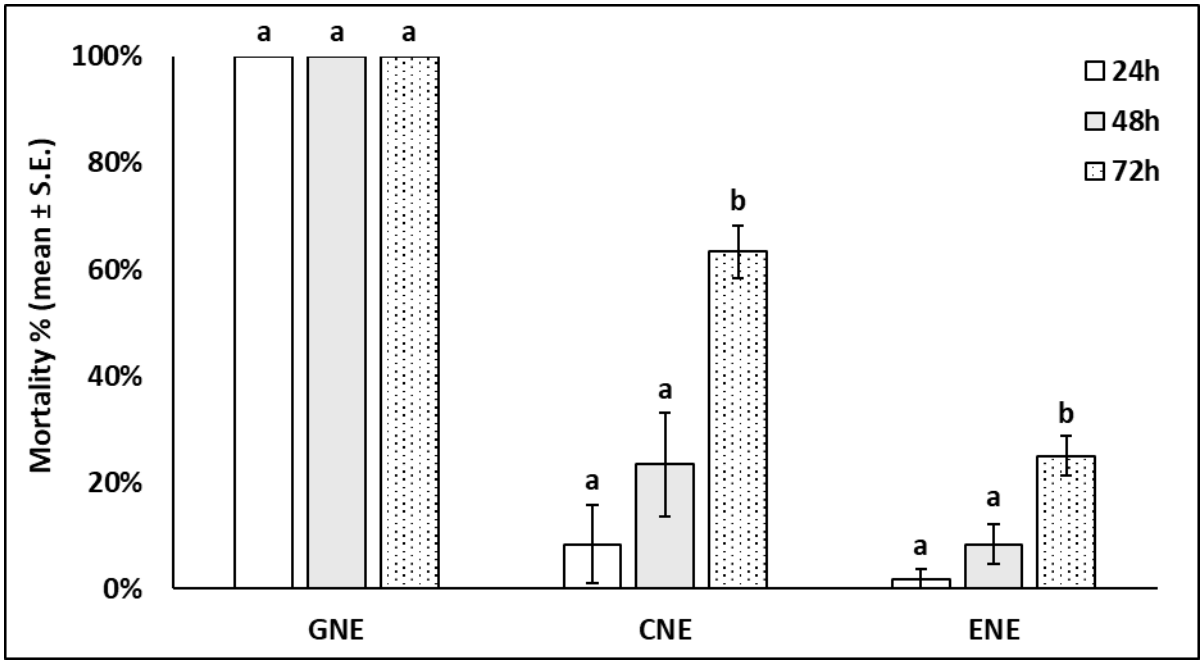


Table 2. Estimated LD_{50} and LD_{90} of GNE against second instar of *D. aberiae* at 24, 48, and 72 hours after exposure.

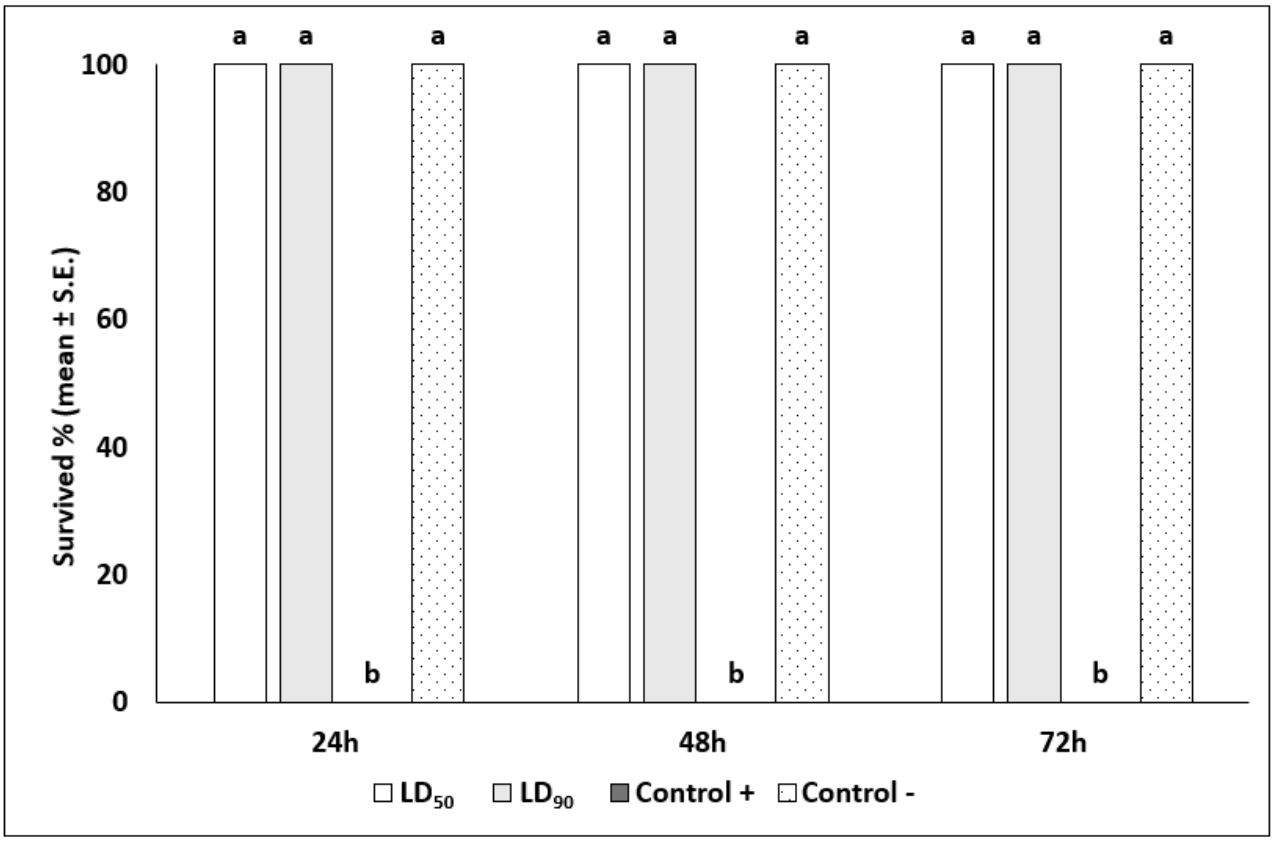
LD[1]	Time (h[2])	Estimated	Lower Bound	Upper bound	*X* (df[3])	*P* level
LD_{50}	24	1.49	0.78	6.11	5.973 (3)	0.113
	48	1.29	1.06	1.56	4.507(3)	0.212
	72	1.14	0.93	1.36	1.899(3)	0.549
LD_{90}	24	2.80	1.79	6.03	5.973 (3)	0.113
	48	2.57	2.00	4.59	4.507(3)	0.212
	72	2.20	1.75	3.50	1.899(3)	0.549

[1]Lethal dose; [2]hours; [3]degrees of freedom. Values were considered statistically different if their 95% fiducial limits did not overlap.

3.3 Side-effects on *Cryptolaemus montrouzieri*

The biological activity of the selected EO-based nano-emulsion (GNE) on the non-target predator *C. montrouzieri* is presented in Fig. 3. Adults were unaffected by GNE, with no mortality recorded at any observed times (24, 48, and 72 hours). Furthermore, no significant differences ($P > 0.05$) were observed between the LD_{50}, LD_{90} (1.14 and 2.20% of EO, respectively) and the negative control (water). In contrast, the positive control (deltamethrin) exhibited high toxicity towards non-target insect adults, with no survivors observed just 24 hours after treatment.

Figure 3. Survival % (mean ± SE) of *C. montrouzieri* adults 24, 48, and 72 hours after exposure to lethal doses (LD_{50} and LD_{90}) of GNE, the negative control (water), and the positive control (deltamethrin). Different letters indicate statistical differences among the treatments within the same exposure time ($P < 0.05$).

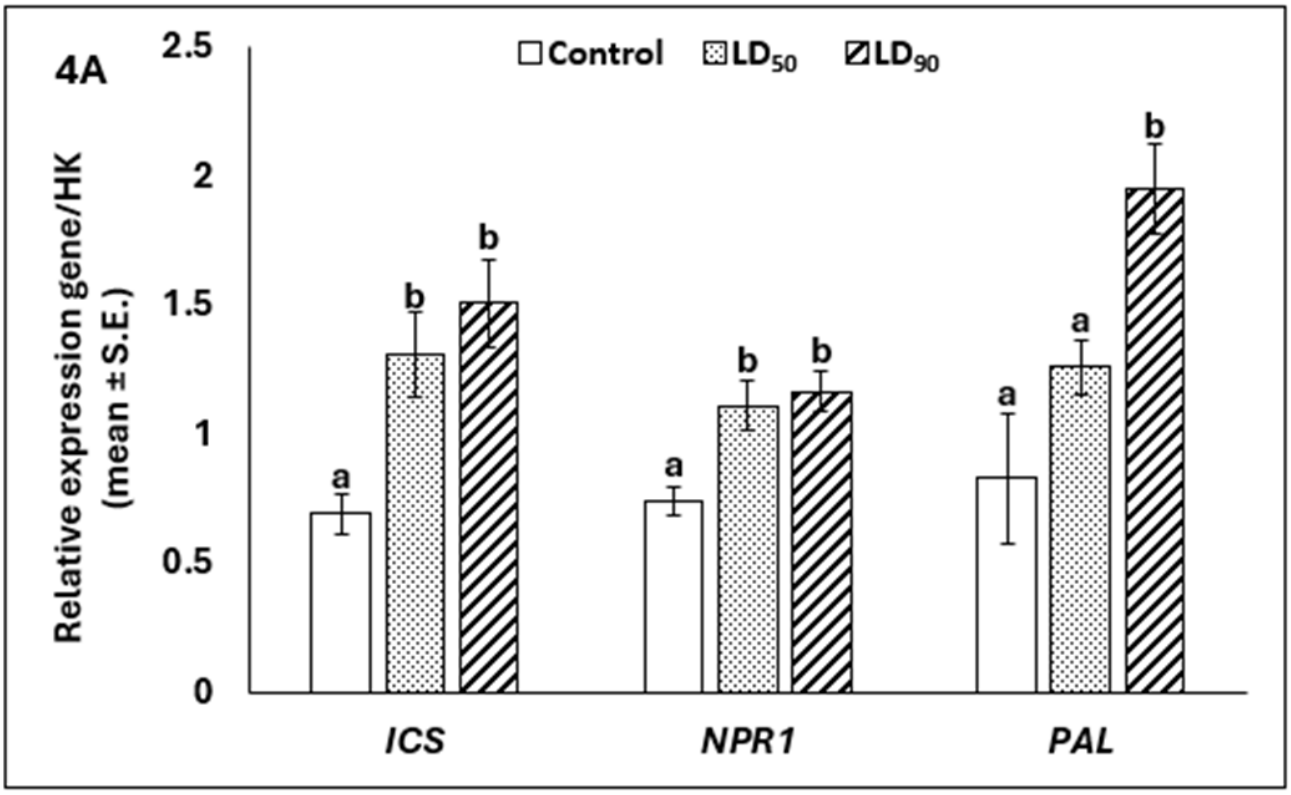


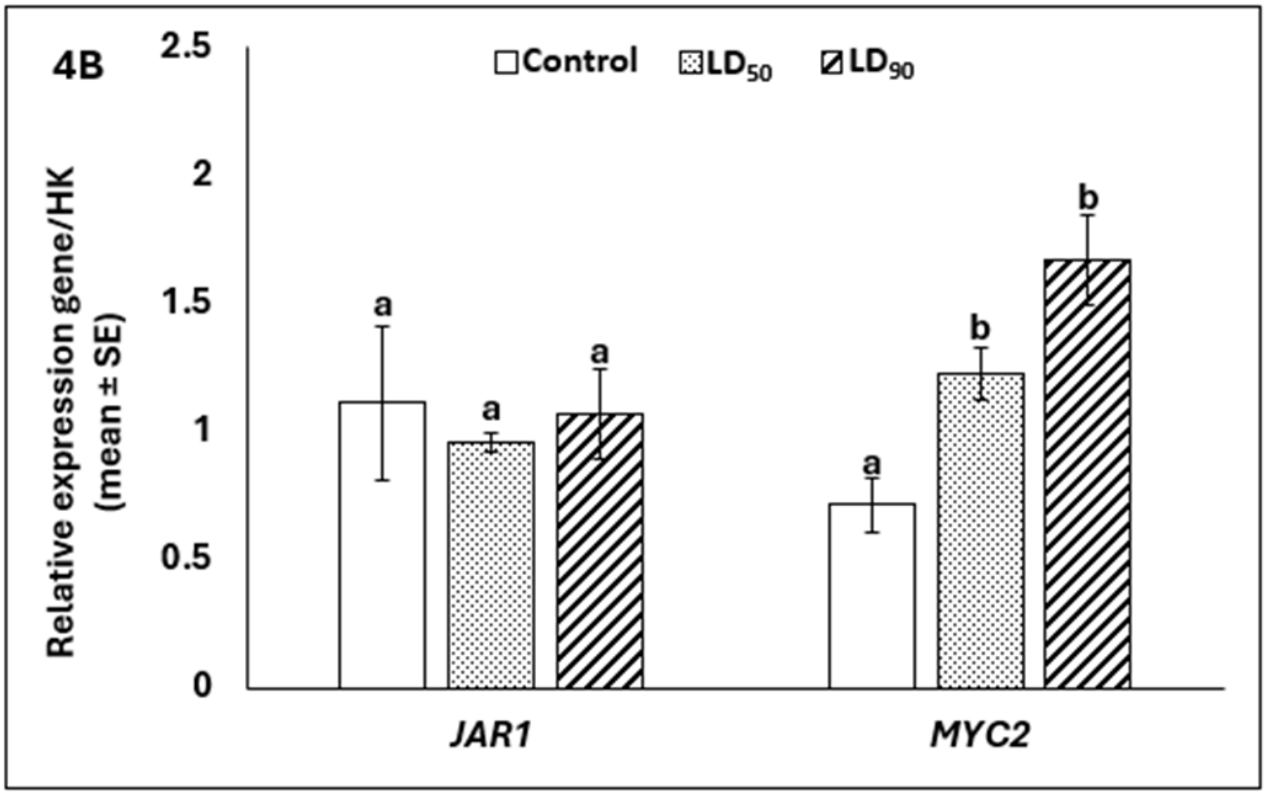
3.4 Phytotoxicity and plant defenses on citrus seedlings

No phytotoxicity (*Pi*= 0) was observed in *C. citrange* plants at any of the tested times (24h, 3d, 7d, and 14 d) across the tested doses (LD_{50} and LD_{90}). The capacity of GNE to enhance plant defenses in citrus seedlings is shown in Fig. 4A and 4B. Transcriptional analysis revealed that the GNE application at the estimated LD_{50} (1.14% EO) and LD_{90} (2.20% EO) doses significantly increase the

relative expression levels of *ICS2*, *NPR1*, *PAL* associated with the SA pathway, as well as *MYC2*, associated with the JA pathway. Specifically, significant differences were observed for ICS2 (F = 8.94; df = 2; P < 0.01) and *NPR1* (F = 8.65; df = 2; P < 0.01) among the control treatment (0.69 ± 0.07 and 0.74 ± 0.06 for *ICS2* and *NPR1*, respectively), LD_{50} treatment (1.31 ± 0.16 and 1.11 ± 0.09 for *ICS2* and *NPR1*, respectively) and LD_{90} treatment (1.51 ± 0.17 and 1.17 ± 0.08 for *ICS2* and *NPR1*, respectively). No significant differences, however, were found bet1.14ween the LD_{50} and LD_{90} doses. In the *PAL* gene experiment, only LD_{90} (1.95 ± 0.18) significantly increased the relative expression level compared to the control (F = 9.05; df= 2; P < 0.01). The *MYC2* gene, involved in the JA pathway, also shows significant differences between the lethal doses (LD_{50} = 1.22 ± 0.1 and LD_{90} = 1.67 ± 0.17) and the control (0.71 ± 0.1) (F = 13.12; df = 2; P < 0.001). Conversely, *JAR1* gene expression showed no statistical differences across the treatments (P > 0.05).

Figure 4. Relative expression of HK/gene (mean ± SE) for *ICS2*, *NPR1,* and *PAL* (4A) and *JAR1* and *MYC2* (4B) in the apical portion of *C. citrange* seedlings 24 hours after treatment with control (water), $LD_{50,}$ and LD_{90} of GNE. Transcript levels were normalized to the expression of the housekeeping gene (*GADPH*). Different letters indicate statistical differences among treatments within the same gene (P < 0.05).




4B
Control
LD50
LD90
Relative expression gene/HK (mean ± SE)
2.5
2
1.5
1
0.5
0
a
a
a
a
b
b
JAR1
MYC2

4. DISCUSSION

This study aimed to develop highly stable EO-based nano-emulsions (i.e., GNE, CNE, and ENE) and to investigate their toxicity against *D. aberiae*. Although the stability of nano-emulsions is influenced by the chemical profile of the essential oil (EO), this chemical composition of EOs can also be affected by various factors, including environmental conditions, plant parts used, extraction and drying methods, and analytical techniques (Campolo et al., 2020a; Li et al., 2015), our results are consistent in terms of the chemical composition and relative abundance of the different compounds, in agreement with those reported by other authors (Figueiredo et al. 2008; Barra et al., 2010; Dziri et al. 2014; Mossa et al., 2018; Li et al. 2020; Selles et al., 2020; Pant et al. 2021; Modafferi et al. 2024b). This analytical consistency suggests that nano-emulsion production could be feasible across various EO sources. Such reproducibility strengthens the potential for scalable nano-emulsion formulations using diverse plant-derived EOs, broadening the range of sources that could be employed effectively.

The use of nanotechnology to enhance the biological efficacy of botanical-based insecticides is rapidly expanding, with various approaches (e.g., low or high-energy ones) playing a crucial role in achieving high-stable EO-based nano-emulsion (Donsì and Ferrari, 2016; Singh and Pulikkal, 2022). In this study, the EO-based nano-emulsions were developed using the high-energy approach, specifically high-pressure microfluidisation, which is generally favored over low-energy techniques due to its ease of production, scalability for industrial applications and superior physical properties (Barradas and de Holanda e Silva, 2020; Kumar et al., 2025; Tanuku et al., 2024). Supporting these findings, Modafferi et al. (2024b) recently demonstrated that high-energy approaches like sonication and microfluidisation produced garlic EO-based nano-emulsions with particle sizes under 200 nm, maintaining stability throughout a 100-day testing period. In contrast, the self-emulsification technique yielded unstable nano-emulsions, with particle sizes exceeding 500 nm. In our study, the physical characterization of EO-based nano-emulsions indicated outstanding stability up to one year post-development, with particle sizes consistently within the nanoscale range (215.7 ± 2.4, 268 ± 2.5, and 230.5 ± 0.7 for GNE, CNE, and ENE, respectively) and optimal PDI values (always ≤ 0.22). Additionally, the negative surface charge values observed (-30.6, -21.8, and -35.9 for GNE, CNE, and ENE, respectively) contributed to enhanced surface adherence and robust repulsive properties (steric and electrostatic), which further supported long-term stability (Akbari and Nour, 2018; Campolo et al., 2020a). These findings align with previous studies that underscore the efficacy of high-energy methods in producing EO-based nano-emulsions (Kaur et al., 2019; Liu

et al., 2022; Ricupero et al., 2022; Shahavi et al., 2016). For instance, Giuliano et al. (2024) successfully obtained a GNE with optimal particle size and PDI values (141.0 ± 1.37 nm and 0.146 ± 0.009, respectively) through the microfluidisation technique. Similarly, Das et al. (2024) employed sonication to produce CNE with a smaller particle size (approximately 125.3nm) than our nano-emulsion (161.3 ± 0.7nm). However, this method resulted in a notably higher PDI (0.46), indicating less stability over time. Additionally, the sonication method applied by Sugumar et al. (2014) yielded extremely small particles for ENE (ranging from 20 to 40nm) with exceptionally low PDI values (0.06). However, this formulation required twice as much surfactant relative to the EO (ratio 2:1). In contrast, our study prioritized minimizing surfactant usage to mitigate potential phytotoxicity effects on treated plants (Appah et al., 2020; Falk et al., 1994; Temple and Hilton, 1963). In this regard, we opted for a higher EO-to-surfactant ratio (3:1) in our formulations, balancing stability with reduced surfactant concentration. This approach addressed toxicity concerns and maintained desirable physical characteristics in our nanoemulsions. Our results suggest that this formulation strategy, emphasizing high EO content and reduced surfactant usage, may be advantageous for applications where plant health is a priority.

To our knowledge, this study demonstrated, for the first time, the efficacy of EO-based nano-emulsion (GNE) against *D. aberiae*. Although nano-formulations have shown effectiveness against various pests, including crop pests, stored product pests, and blood-feeding insects, their biological activity against scale insects has been underexplored (Campolo, 2022; Esmaili et al., 2021; Menossi et al., 2021; Modafferi et al., 2024a). A limited number of studies have reported on the efficacy of GNE against *P. citri*. For instance, Modafferi et al. (2024c) demonstrated high insecticidal activity of GNE in both topical and residual toxicity assays against the second instar of *P. citri* with LD_{50} and LD_{90} values estimated at 0.78% and 1.08% EO for residual application and 0.24% and 0.96% EO for topical application, respectively, 48 hours after treatment. Similarly, Modafferi et al. (2024b) showed that three GNEs developed using high-energy methods (sonication and microfluidisation) were effective against *P. citri*, with an LD_{90} consistently below 1.5% EO. While our results (LD_{50} = 1.14% EO; LD_{90} = 2.20% EO after 72 hours) are slightly less effective than those reported by Modafferi et al. (2024b), they still underscore the potential of GNE against mealybugs.

One of the most promising attributes of botanical insecticides, whether formulated into nano-delivery systems like GNE or used as conventional EOs, is their potential selectivity towards non-target organisms (e.g., predators, parasitoids, pollinators, plants, and invertebrates), which is crucial for their practical application in real-world settings. In this context, our study confirms the

safety of GNE for the non-target organism *C. montrouzieri*. However, the impact of EOS - whether used in their natural form or nano-formulated - on non-target organisms still needs to be investigated. Some authors have reported minimal impact of *Mentha pulegium* EO, citrus EO, and a D-limonene-based formulation on the survival of *C. montrouzieri* larvae and adults (Attia et al., 2022; Bibi et al., 2022; El Aalaoui et al., 2019). Conversely, *Thymus capitatus* EO has shown substantial toxicity, with *C. montrouzieri* adult mortality reaching about 90% (Alloui-Griza et al., 2022). The potential negative effects of natural substances on non-target insects are influenced by various factors, including the type of plant and insect species involved, exposure route, or application rate (Gahukar and Das, 2020; Giunti et al., 2022). Data on the effects of garlic (whether as aqueous extract or EO) on non-target organisms are limited. Among the few studies available, Fand & Kamra (2012) evaluated garlic aqueous extract at concentrations of 0.5, 1, and 1.5 % against *C. montrouzieri* larvae, observing a mortality rate of less than 20% at 72 hours post-exposure. Similarly, Modafferi et al. (2024) found no toxicity to *C. montrouzieri* adults in topical toxicity trials using a garlic EO-based nano-emulsion.

On the other hand, the adverse effects of EOs on plants, particularly phytotoxicity, are well documented. Several studies have reported that EOs can inhibit photosynthesis and mitochondrial respiration, induce genotoxic effects, interfere with enzymes and phytohormones, alter membrane properties, and promote the formation of reactive oxygen species (ROS) (De Almeida et al., 2010; Pinheiro et al., 2015; Verdeguer et al., 2020; Werrie et al., 2020). However, encapsulating EOs in nano-delivery systems appears to mitigate these negative effects. For instance, various studies have shown that botanical-based nano-formulations (such as EO-based nano-emulsions and/or nanoparticles) did not produce adverse effects on plants like garlic, tomato, and maize (Campolo et al., 2020b; Qian et al., 2023). Similarly, Pasquoto-Stigliani et al. (2017) noted that the adverse effects of botanical nano-insecticides on garlic and maize plants were dose-dependent. In the case of GNE, previous studies reported its safe application on tomato plants, with *Pi* values comparable to those observed in *C. citrange* seedlings in our research (*Pi* = 0) (Ricupero et al., 2022). Likewise, Giuliano et al. (2024) observed that GNE application on pepper plants (*Capsicum annuum* L., cv Makko F1) did not affect the growth, leaf number, weight, and chlorophyll content. However, mild phytotoxic effects were noted on the leaves, with low *Pi* levels (0.13 ± 0.01), and a significant reduction in the number of fruits was observed compared to the control.

One of the most promising aspects of using EOs or their nano-formulations for crop protection is their potential to enhance natural plant defenses. Studies have shown that applying EOs can boost

defense enzyme activities (e.g., chitinase, 1,3-β-glucanase, phenylalanine ammonia-lyase, and peroxidase) (Kaneko et al., 2024; Kesraoui et al., 2022). Additionally, EO treatments can induce systemic resistance by over-expressing genes involved in plant defense (Andres et al., 2024; Sellamuthu et al., 2013; Werrie et al., 2022). In our study, GNE increased the expression levels of several genes involved in the SA and JA pathways. Previous research showed that the foliar application of *Achillea millefolium* and *Allium sativum* EOs provided a dual benefit on tomato plants (Ben Abdallah et al., 2023), leading to a decrease in *Phthorimaea absoluta* (Meyrick) (Lepidoptera: Gelechiidae) populations and an increase in the expression levels of proteinase inhibitor II (*PIN2*) and abscisic acid stress ripening 1 (*ASR1*), associated with the JA and ABA signaling pathways, respectively. Similarly, Ricupero et al. (2023) showed that garlic and peppermint EOs increased the expression levels of the basic PR-1 protein precursor (*PR1*) and *PIN2* compared to the control treatment.

5. CONCLUSION

In conclusion, this study demonstrates, for the first time, the efficacy of botanical-based nano-insecticides, specifically GNE, against *D. aberiae*. The high-pressure microfluidisation technique enabled the development of a highly stable formulation with optimal physical properties featuring a high EO-to-surfactant ratio (3:1) that prevented phytotoxic effects on citrus seedlings. Additionally, the solid insecticidal activity against the target pest and the absence of side effects on the non-target coccinellid predator *C. montrouzieri* supports its potential integration into IPM programs. The overexpression of defense-related genes in the SA and JA pathways further suggests an additional plant-protective effect, enhancing resilience against pest damage. However, to ensure the safe application of GNE and similar formulations, further research is necessary to investigate their toxicological impacts on a broader range of non-target organisms, including pollinators, beneficial invertebrates, and aquatic organisms. Future studies should also explore the efficacy of these nano-formulated botanical insecticides against other economically significant plant pests, both in laboratory settings and under field conditions.

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SUPPLEMENTARY MATERIALS

Supplementary Table 1: Primer sequences.

Gene	Gene description	Primer sequence (5'-3')
PAL	Phenylalanine Ammonia-Lyase	FW: CACATTCTTGGTAGCGCTTTG RV: AGCTACTTGGCTGACAGTATTC
ICS2	Isochorismate Synthase 2, Chloroplastic	FW: GGAGGAGGAGAGAGTGAATTTG RW: GGGTTGCTTCCTTCTACTATCC
NPR1	Non-Pathogenesis Related Protein 1	FW: GTACCTTGAAAACAGAGTTGGACTGG RW: TGCTCCTCTTGCATTTTGAAAGGTG
JAR1	Jasmonate Resistant 1	FW: AAGGCGATGCAGTCACAATG RW: TGGTGGAAATCAGGACCAAAG
MYC2	Transcription factor MYC2, DNA binding protein	FW: TGCATCTACAGCCGACCC RW: TAGGTCCAGCCCTCACGA
GADPH	Glyceraldehyde-3-phosphate dehydrogenase	FW: GGAAGGTCAAGATCGGAATCAA RW: CGTCCCTCTGCAAGATGACTCT

Supplementary Table 2: Chemical composition of *Allium sativum* EO.

Component	LRI[1]	LRI[2]	RT[3]	Area (%)
Allyl isopropyl sulfide	826	826	3.25	0.01
1,2-Dithiolane	841	842	3.49	0.59
Diallyl sulfide	856	850	3.73	6.21
Allyl propyl sulfide	871	875	3.95	0.01
Methyl isopropyl disulphide	895	899	4.33	*[4]
Allyl methyl disulfide	917	919	4.84	2.54
Dimethyl trisulfide	970	970	6.18	0.02
1,3-Dithiane	1021	1027	7.67	0.01
Allyl propyl disulfide	1049	1048	8.64	0.34
Diallyl disulphide	1084	1082	9.83	32.32
2-Propenyl propyl disulfide	1094	1097	10.19	0.17
Allyl methyl trisulfide	1138	1135	11.89	2.40
4-Methyl-1,2,3-trithiolane	1151	1150	12.43	2.54
3-Vinyl-1,2-dithiacyclohex-4-ene	1186	1180	13.81	0.12
2-Vinyl-4H-1,3-dithiine	1211	1215	14.84	0.48
Dimethyl tetrasulphide	1215	1215	14.99	*
Allyl isopropil trisulfide	1264	1266	17.08	0.28
Diallyl trisulfide	1303	1300	18.69	26.83
Allyl propyl trisulfide	1312	1307	19.09	0.07
5-Methyl-1,2,3,4-tetrathiane	1358	1359	21.00	1.03
Diallyl tetrasulphide	1540	1540	28.37	13.56
6-Methyl-4,5,8-trithia-1,10-undecadiene	1591	1591	30.36	0.86
8-Methyl-4,5,6,9-tetrathia-1,11-dodecadiene	1812	1815	38.34	7.47
Sulfur components		**97.87%**		
Total identified		**97.87%**		

[1]Calculated linear retention index; [2]Litterature linear retention index; [3]Retention time; [4]<0.01%.

Supplementary Table 3: Chemical composition of *Syzygium aromaticum* EO.

Component	LRI[1]	LRI[2]	RT[3]	Area (%)
Furfural	831	831	3.35	0.03
α-Thujene	933	932	5.25	0.01
Camphene	947	947	5.62	*[4]
α-Sabinene	976	978	6.36	*
o-Cymene	1024	1027	7.78	0.01
Limonene	1028	1028	7.91	0.02
Eucalyptol	1031	1031	8.02	0.04
2-Methoxy-phenol	1089	1090	10.03	0.01
Linalool	1101	1100	10.44	0.02
4,8-Dimethyl-1,3,7-nonatriene	1116	1114	11.06	0.01
Camphor	1144	1146	12.17	*
α-Terpineol	1191	1190	14.05	*
Methyl salicylate	1194	1193	14.17	0.16
4-(2-propenyl)-phenol	1256	1251	16.75	0.19
Eugenol	1365	1359	21.34	81.72
α-Copaene	1376	1377	21.77	0.31
Vanillin	1399	1399	22.73	0.05
Methyleugenol	1407	1410	23.06	0.10
Caryophyllene	1419	1418	23.54	6.97
Humulene	1452	1451	24.91	1.00
α-Muurolene	1500	1499	26.83	0.01
(+)-δ-Cadinene	1523	1524	27.74	0.40
Acetyleugenol	1530	1525	28.03	6.58
Caryophyllenyl alcohol	1569	1568	29.52	0.04
β-Caryophyllene oxide	1582	1583	30.03	1.65
Humulene epoxide	1608	1605	31.04	0.14
Aldehydes		**0.03%**		
Monotepenes		**0.05%**		
Oxygenated monoterpenes		**0.06%**		
Phenylpropenes		**88.65%**		
Esters		**0.16%**		
Sesquiterpenes		**8.69%**		
Oxygenated sesquiterpenes		**1.83%**		
Total identified		**99.45%**		

[1]Calculated linear retention index; [2]Litterature linear retention index; [3]Retention time; [4]<0.01%.

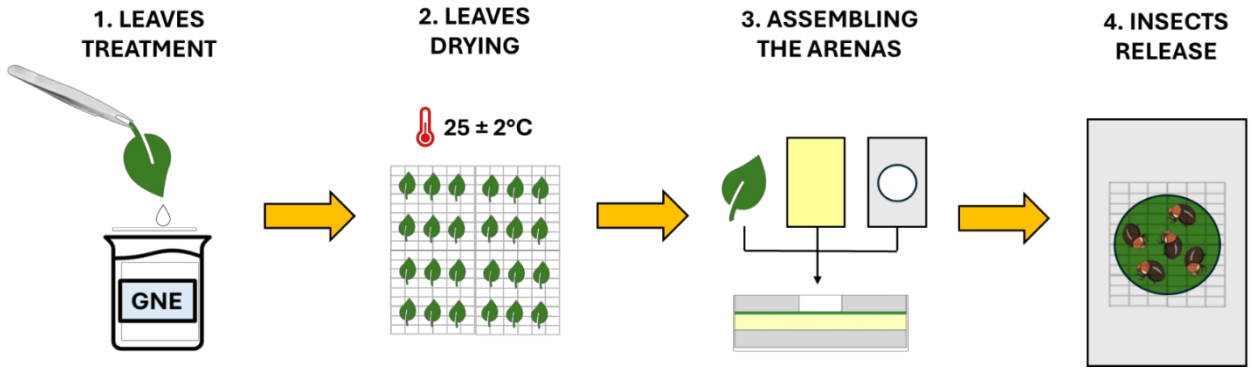
Supplementary Table 4: Chemical composition of *Eucalyptus camaldulensis* EO.

Compounds	LRI[1]	LRI[2]	RT[3]	Area (%)
Hexanal	801	802	2.86	0.01
Isoamyl acetate	872	876	3.97	*[4]
α-Thujene	925	926	5.04	1.65
α-Pinene	932	933	5.22	3.07
2,4(10)-Thujadien	942	945	5.47	0.63
Camphene	947	947	5.59	0.02
Verbenene	952	951	5.72	0.11
Sabinene	972	973	6.22	0.52
β-Pinene	976	980	6.32	0.80
β-Myrcene	990	992	6.68	0.52
2-Carene	1002	1003	6.99	0.03
α-Phellandrene	1005	1006	7.11	1.44
α-Terpinene	1017	1019	7.49	0.86
p-Cymene	1027	1027	7.85	29.42
β-Phelladrene	1030	1033	7.95	5.43
Eucalyptol	1032	1035	8.03	7.92
Thujol	1047	1039	8.53	0.03
γ-Terpinene	1057	1056	8.89	0.91
4-Thujanol-trans	1066	1067	9.19	0.02
Linalool oxide	1071	1074	9.38	0.14
ρ-Cymenene	1089	1090	9.98	0.79
2-Nonanone	1092	1092	10.1	0.03
Linalool	1100	1101	10.38	0.57
Hotrienol	1105	1104	10.55	0.09
β-Thujone	1117	1114	11.03	0.43
ρ-Menth-2-en-1-ol	1121	1120	11.21	1.04
α-Campholenal	1126	1127	11.39	0.22
2,6-Dimethyl-1,3,5,7-octatetraene, e,e-	1134	1130	11.74	0.10
ρ-Menth-3-en-1-ol	1139	1139	11.91	0.92
Sabina ketone	1158	1158	12.67	0.13
Borneol	1168	1168	13.08	0.34
Terpinen-4-ol	1178	1177	13.47	4.99
Cryptone	1189	1188	13.92	11.33
α-Terpineol	1191	1191	14.01	0.37
Piperitol-cis	1196	1195	14.19	0.10
Myrtenal	1197	1197	14.23	0.08
Piperitol-trans	1208	1208	14.68	0.23
Verbenone	1210	1207	14.78	0.05
Carveol-trans	1219	1220	15.15	0.14

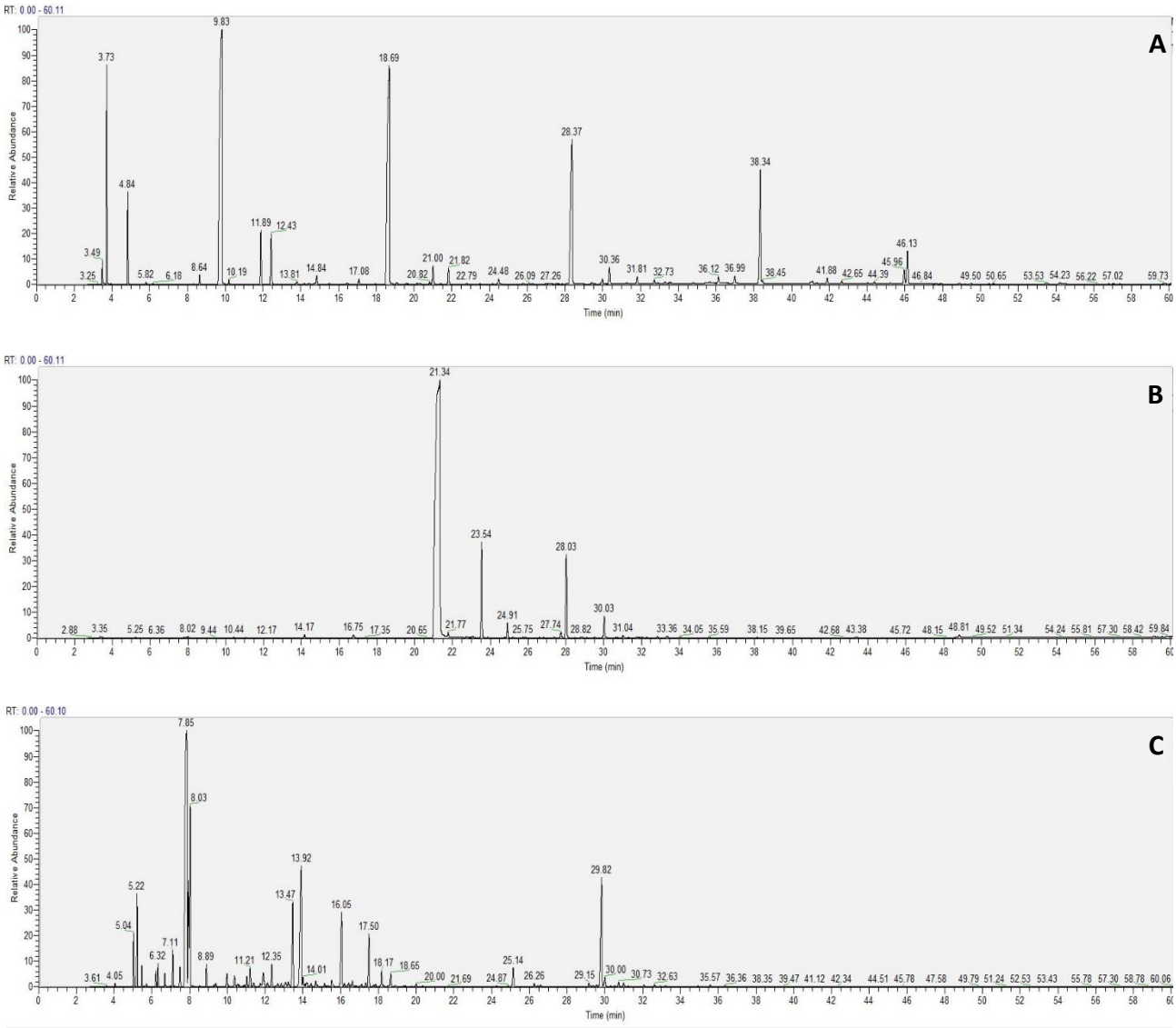
ρ-Cumenol	1228	1221	15.53	0.32
Cumaldehyde	1240	1240	16.05	4.54
Carvone	1244	1244	16.20	0.15
(+)-Carvotanacetone	1247	1247	16.35	0.04
Piperiton	1254	1254	16.62	0.32
Phellandral	1275	1274	17.50	3.06
ρ-Cymen-7-ol	1290	1289	18.17	0.78
Thymol	1293	1292	18.26	0.02
Carvacrol	1302	1300	18.65	0.63
ρ-Mentha-1,4-dien-7-ol	1329	1332	19.76	0.03
Neryl acetate	1366	1367	21.32	0.01
α-Copaene	1375	1377	21.69	0.02
β-Elemene	1391	1392	22.39	0.02
α-Gurjunene	1408	1410	23.07	0.01
β-Gurjunene	1437	1432	24.26	0.02
Dehydroaromadendrene	1449	1450	24.75	0.01
Alloaromadendrene	1459	1457	25.14	1.20
Elixene	1495	1492	26.60	0.07
α-Muurolene	1499	1499	26.77	0.01
γ-Cadinene	1513	1513	27.30	0.02
Cubenene	1523	1522	27.69	0.03
(+)-Spathulenol	1578	1578	29.82	7.94
(-)-Spathulenol	1582	1582	30.00	0.60
Viridiflorol	1590	1590	30.30	0.02
Aldehydes	**0.01%**			
Monotepenes	**61.03%**			
Oxygenated monoterpenes	**17.56%**			
Phenylpropenes	**6.76%**			
Esters	**0.01%**			
Sesquiterpenes	**1.40%**			
Oxygenated sesquiterpenes	**8.56%**			
Total identified	**95.33%**			

[1]Calculated linear retention index; [2]Litterature linear retention index; [3]Retention time; [4]<0.01%.

Supplementary Fig. 1: Schematic representation of bioassay towards non-target insect.



Supplementary Fig. 2: *Allium sativum* (A), *Syzygium aromaticum* (B) and *Eucalyptus camaldulensis* (C) EOs GC-MS chromatograms.



CHAPTER 7

Metabolic and microbial responses of *Ceratitis capitata* to essential oil-based nanoemulsions: implications for pest management

Metabolic and microbial responses of *Ceratitis capitata* to essential oil-based nanoemulsions: implications for pest management

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ABSTRACT

Growing concerns about the environmental and health impacts of synthetic pesticides have led to stricter regulations and a shift toward eco-friendly pest management. Essential oils (EOs) are a promising alternative, but challenges like high volatility, poor water solubility, and rapid degradation limit their use in Integrated Pest Management (IPM). To overcome these limitations, this study aimed to develop garlic, eucalyptus, and clove EO-based nano-emulsion (EO-NEs) in a bait treatment format through the high-pressure microfluidization technique. The biological activity against *Ceratitis capitata* and towards the parasitoid *Anagaspis daci* was evaluated through ingestion toxicity trials. The garlic nano-emulsion (garlic-NE) with estimated LD_{50} and LD_{90} of 0.96 and 2.18 % of EO showed high insecticidal activity against *C. capitata*. However, garlic-NE exhibited high insecticidal activity (100% mortality) towards the parasitoid *A. daci*. We analyzed the expression of 14 genes involved in detoxification pathways to investigate the mechanisms underlying garlic-NE toxicity. Our results showed that garlic-NE triggered a strong metabolic detoxification response, leading to the upregulation of cytochrome P450 monooxygenases (CYPs), glutathione S-transferases (GSTs), and alcohol dehydrogenase (Adh). Furthermore, garlic-NE

induced significant shifts in the gut microbiota composition of *C. capitata*, disrupting its microbial homeostasis. This dual effect—metabolic stress combined with microbiota disruption—suggests that garlic-NE exerts its insecticidal action through a multifaceted mode of action rather than direct neurotoxicity, potentially reducing the risk of resistance development. Overall, this study highlights the potential of EO-based nano-formulations as sustainable insecticides.

KEYWORDS: *Ceratitis capitata*; botanicals; nanotechnology; biopesticides; non-target organisms; detoxification system

1. INTRODUCTION

Ceratitis capitata (Wiedemann) (Diptera: Tephritidae), commonly known as the Mediterranean fruit fly, is a significant global pest affecting many fruit crops. Its infestations cause substantial economic losses by reducing fruit quality and marketability (Blythe et al., 2020; Giunti et al., 2023a; Liquido et al., 1990; Mavrikakis et al., 2000). Despite significant advancements in integrated pest management (IPM) strategies and the adoption of more sustainable methods in certain regions, such as biological control, the use of bio inputs, and the Sterile Insect Technique (SIT), chemical control remains the primary approach for managing this pest in many parts of the world. However, while effective, the reliance on synthetic pesticides presents several challenges, including the development of resistance, environmental contamination, and adverse effects on non-target organisms, including humans and beneficial insects (Laudani et al., 2022; Urbaneja et al., 2009).

Over the last decade, innovative and eco-friendly pest management strategies have been investigated to overcome these challenges for their potential application against *C. capitata* (Giunti et al., 2023b; Ortolá et al., 2024). Using natural antagonists such as predators and parasitoids represents a promising approach. Indeed, different authors reported the prey activity of different species of ants, carabids, wasps, spiders, and others (Garcia et al., 2020; Hendrichs et al., 1994; Urbaneja et al., 2006). In addition, more than 60 parasitoids belonging to Braconidae, Chalcididae, Diapriidae, Eulophidae, Figitidae, and Pteromalidae families were reported as medfly parasitoids (Giunti et al., 2023b). Despite the large number of predators and parasitoids, biological control alone cannot reduce the huge damage to fruits caused by this pest (Giunti et al., 2023b).

Among the green control strategies to manage *C. capitata*, the use of botanicals, in particular essential oils (EOs), as insecticides is considered an innovative strategy (Campolo, 2022). In this context, several authors reported the efficacy of these substances against the Medfly. Benelli et al. (2012) studied the ingestion, topical, and fumigation activity of different EOs, highlighting a good insecticidal activity, mainly through topical application, with a mortality rate of around 70% at 0.1µL/fly for *Rosmarinus officinalis* L., *Lavandula angustifolia* Millerand, and *Thuja occidentalis* L.. Similarly, Miguel et al. (2010) investigated the biological activities of three EOs (*Mentha pulegium*, *Thymbra capitata*, and *Thymus albicans*) against *C. capitata* adults, showing a high insecticidal activity of *M. pulegium* EO with a mortality rate of 90%. The toxicity of EOs was also proven against *C. capitata* larvae. For instance, through ingestion toxicity trials, Papachristos et al. (2009) highlighted good insecticidal activity for different citrus EOs.

The efficacy of the EOs can be attributed to their ability to interfere with some physiological traits for insect viability (Priestley et al., 2003; Zhou et al., 2008). For instance, EOs are known to affect the nervous systems of insects (Jankowska et al., 2018). In this context, insects treated with EOs inhibited the acetylcholinesterase (*AchE*) activity (Abdelgaleil et al., 2009; Seo et al., 2014; Yeom et al., 2015). In addition, the inhibitory activities of EOs on octopamine and GABA receptors and transient receptor potential (TRP) channels were also confirmed (Blenau et al., 2012; Mossa, 2016; Shaaya and Rafaeli, 2007). Another important aspect related to the efficacy of the EOs as insecticides is their presumed ability to negatively affect the gut microbiome of the insects (Dehghankar et al., 2021; Szczepanik et al., 2018). Akami et al. (2019b), for example, highlighted that *Lippia adoensis* EO affects the richness, diversity, and abundance of Proteobacteria, Firmicutes, and Bacteroidetes on *Callosobruchus maculatus* (F.) (Coleoptera: Chrysomelidae). The insect gut microbiome plays a crucial role in digestion, immune system function, and detoxification of xenobiotics, influencing insecticide resistance (Engel & Moran, 2013; Douglas, 2015). Disrupting these microbial communities could compromise the insect's ability to metabolize toxic compounds, affecting digestion, nutrient absorption, and overall resilience to chemical stressors. Therefore, investigating the interplay between metabolic detoxification pathways and microbiome alterations is essential to understanding the mode of action of novel insecticidal compounds such as essential oil-based nanoemulsions (EO-NEs).

Despite the promising results of the EOs as insecticides, their practical application is a challenge due to some negative characteristics of EOs, such as volatility, low water solubility, rapid degradation under environmental conditions, and flammability (Campolo, 2022; Modafferi et al., 2024c). However, in the last decade, the use of nanotechnology to overcome these challenges increased the interest of the different stakeholders (Campolo, 2022; Koul, 2019). Nanotechnology allows nano-formulations characterized by unique chemical, physical, and biological attributes. These include: (i) enhanced water solubility, (ii) improved surface coverage, (iii) gradual release of active ingredients (a.i.), (iv) reduced degradation of a.i., (v) minimized phytotoxic effects, (vi) increased permeability through insect cuticles, and (vii) boosted bioactivity (Campolo, 2022; Pavoni et al., 2020). Botanical-based nano-formulations used for insecticidal applications can be achieved through two main approaches, namely bottom-up (i.e., self-emulsification process, phase inversion temperature, etc.) and top-down (i.e., sonication, high-pressure homogenization, and microfluidization) (Donsì and Ferrari, 2016; Nirmala et al., 2022). Despite several authors reporting the efficacy of both approaches, top-down methods are generally preferred due to some

advantages such as industrial scalability, low amount of co-formulants used in the formulations, and optimal physical characteristics (i.e. size, PDI, and surface charge) and stability of developed nano-formulation (Campolo et al., 2020a; Modafferi et al., 2024b).

In this context, this study aimed to develop three different EO-NEs (garlic-NE, eucalyptus-NE, and clove-NE) through a top-down approach (microfluidization) using a high EO: surfactant ratio (3: 1) and to investigate the biological activity against *C. capitata* adults. The physical properties (particle size, PDI, and surface charge) of EO-NEs were assessed through dynamic light scattering (DLS) analysis. The efficacy of the developed EO-NEs was evaluated against the target insect through ingestion trials when applied in bait formulations to simulate practical field use conditions and assess their potential applicability. At the same time, the side effects of the most effective EO-NE (garlic-NE) against the target pest were evaluated towards the larval-pupal parasitoid *Anagaspis daci* (Weld) (Hymenoptera: Figitidae) (de Pedro et al., 2016). Additionally, the expression of genes involved in different detoxification mechanisms and the effect on the gut microbiome of *C. capitata* were evaluated.

2. Materials and methods

2.1 Insect rearing

Ceratitis capitata and *A. daci* were reared for several generations in the laboratories of the IVIA. Specifically, the Mediterranean fruit fly was reared according to the methods described in Jacas et al. (2008). The colony was maintained under constant climatic conditions (25±2°C, 60-70% RH with a photoperiod of 14h:10h L:D) and was reinforced by regular additions of flies emerging from pupae collected from naturally infested fruits in the field. Adults were fed with a mixture of sugar and hydrolyzed yeast extract (ratio 4:1 w: w) and water *ad libitum*.

Aganaspis daci was reared according to the methods described by de Pedro et al. (2018), and the original colony was obtained from Medfly larvae collected from figs in a nearby Valencian village (Bétera, Spain). Wasp adults were reared in plastic cages (35 x 30 x 35 cm) provided with water, sugar, and honey as food sources under constant climatic conditions (22±2°C, 65±5% RH, and 16h:8h L:D photoperiod). *C. capitata* pupae were used as host species. For both species, newly emerged adults (0-2 days) were used in the experiments.

2.2 Chemical Characterization of EOs

The *Allium sativum* EO (garlic) and *Syzygium aromaticum* EO (clove) were purchased from Esperis S.p.A. (Milan, Italy). *Eucalyptus camaldulensis* EO (eucalyptus) was steam-extracted from leaves collected from plants growing in southern Italy (Reggio Calabria, Italy). The EOs were chemically analyzed through a gas-chromatography/mass-spectrometry (GC-MS) apparatus. A total of 23, 26, and 63 compounds corresponding to 97.87%, 99.45%, and 95.33% of the total area, were detected in garlic, clove, and eucalyptus EOs, respectively. Garlic EO was composed only of sulfur compounds, clove EO was mainly comprised of oxygenated monoterpenes (88.65%), and eucalyptus EO was mainly composed of monoterpenes (61.03%). See Modafferi et al. (2025) for complete analytical procedures and chemical characterizations.

2.3 EO-based nano-emulsions: formulation and characterization

The EO-NEs (garlic, eucalyptus, and clove-NE) were obtained according to the methods described by Modafferi et al. (2024). First, the EO and tween 80® (polyoxyethylene (20) sorbitan monooleate, Sigma Aldrich, Munich, Germany) (ratio 3:1) were mixed for 5 min at 7000 RPM through a magnetic stirrer. Then, double-stilled water was added slowly (1 mL min^{-1}) to the previous solution (EO and surfactant). The obtained emulsion (EO 15% w/w; tween 80® 5% w/w; water 80% w/w) was

homogenized five times using a high-pressure microfluidizer device (HPM) (LM20 Microfluidizer™ Processor, USA) at 30.000 PSI. The resulting EO-NEs were collected in aluminum bottles and stored at 4°C. The EO-NEs were physically characterized through Dynamic Light Scattering (DLS) analysis to assess particle size (nm), the polydispersity index (PDI), and surface charge (ζ-potential). The analyses were performed by diluting the obtained EO-NEs in double distilled water (ratio 1:200) 24 hours after the development.

2.4 Insecticidal activity of developed EO-NEs

2.4.1 *Ceratitis capitata*

The insecticidal activity of the developed EO-NEs was evaluated through ingestion trials against *C. capitata* adults (Juan-Blasco et al., 2013). The desired dilutions were prepared by diluting the required amount of EO-NE in a sucrose-water solution (30% w/v) to simulate the practical conditions under which insecticides are used in bait formulations for pest management programs against *C. capitata* (Chueca et al., 2007). In detail, the trials were carried out inside plastic boxes (5 x 10 x 10 cm) in which ten newly emerged Medflies (sex ratio 1:1) were fed daily with four drops (50 µL each) of the tested formulations until the end of the experiment (72 hours). To evaluate the efficacy of each EO-NE (garlic, eucalyptus, and clove) against the target insect and to select the most effective one, an initial experiment was conducted using a high percentage of active ingredient (a.i.) (2.5% of EO). Bioassays were performed under the same rearing conditions (25 ± 2°C, 60-70% RH with a photoperiod of 14h:10h L:D). Insects were considered dead if they were unable to fly. Eight replicates were performed for each dilution; sucrose water solution (30% w/v) and Spintor™ Fly at label concentration (0.048 g/L) were used as a negative control (C-) and positive control (C+), respectively. This experiment demonstrated that garlic-NE was the most effective formulation, and therefore, it was the only one subjected to a serial dilution bioassay (i.e., 1.87, 1.25, 0.93, 0.625, and 0.46% w: w of EO). The other nano-formulations were discharged, as the concentrations required to control Medfly adults were not viable for practical application.

2.4.2 *Aganaspis daci*

The toxic effects of selected garlic-NE towards *A. daci* adults were evaluated through the above-mentioned ingestion trials, using the estimated LD_{50} (0.96% of EO) and LD_{90} (2.16% of EO) against *C. capitata* adults (see Table 2), sucrose water solution (30% w/v) as negative control (C-) and Spintor™ Fly at label concentration (0.048 g/L) as positive control (C+). Preliminary trials

highlighted no mortality between the sucrose-water and sucrose-water-tween 80 solutions. The trials, replicated ten times, were carried out inside plastic Petri dishes (9 cm Ø) with a hole in the lid (3 cm Ø) sealed with muslin. Five *A. daci* adults were carefully placed inside the arena and fed daily with four drops (50 µL each) of the tested formulation or controls. Arenas were maintained inside a climate chamber at 22 ± 2 °C, 65 ± 5% relative humidity (RH), and 16h: 8h (light: dark L: D) photoperiod. Mortality was recorded 72 hours after treatments. Insects were considered dead if they could not fly or remained immobile when stimulated with a fine brush.

2.5 Effects on detoxification genes of *C. capitata*

To evaluate the effects of garlic-NE on insect detoxification pathways, the transcriptional levels of 13 genes involved in different metabolic processes were analyzed via real-time PCR (qPCR) (Table 1). Three treatments were applied using the estimated **LD_{50}** (0.96% EO) and **LD_{90}** (2.18% EO) against *C. capitata* (see Table 2), along with a negative control (C-). The analyzed genes were categorized based on their roles in detoxification pathways and neurotransmitter metabolism.

- Phase I detoxification (Modification of xenobiotics): This phase involves genes encoding Cytochrome P450 monooxygenases (CYPs) (*Cyp6g2, Cyp6a2, Cyp6a69, Cyp12e1*), which catalyze oxidation reactions to enhance the solubility of xenobiotics, facilitating their subsequent metabolism and excretion. Alcohol Dehydrogenase (Adh) was included, as it plays a crucial role in the oxidation of alcohol-derived compounds, contributing to xenobiotic detoxification.
- Phase II detoxification (Conjugation and solubilization): This phase involves genes encoding Glutathione S-transferases (GSTs) (*GSTS1, MGSTL1*), which conjugate toxic compounds with glutathione, enhancing their solubility and promoting their elimination from the organism.
- Neurotransmitter metabolism and synaptic regulation: Several neurotransmission-related genes were also analyzed. Acetylcholinesterase (AChE) degrades acetylcholine at the synapse, regulating neural signaling. Tyramine beta-hydroxylase (TBH) catalyzes the conversion of tyramine into octopamine, a key neurotransmitter involved in insect neuromodulation. Lastly, Gamma-aminobutyric acid transaminase (GABAT) plays a role in the degradation of gamma-aminobutyric acid (GABA), the primary inhibitory neurotransmitter in insects, which is crucial for maintaining neural homeostasis.

The TUB1a (*tubulin1-alpha*) gene was used as a housekeeping gene for normalization (Ponton et al., 2011). Each treatment was replicated ten times, following the protocol described in section 2.4.1. Twenty-four hours after the treatment, the Medflies were placed inside an Eppendorf (2mL), immediately ground in liquid nitrogen, and then stored at -80°C until the RNA extraction. Following the manufacturer's recommendations, total RNA was extracted using NZYol (NZYTech, Lisboa, Portugal). Then, 10 µg of total RNA was treated with a TURBOTM DNA-free kit (Ambion, Life Technologies, CA, United States) to remove the DNA contamination. Reverse transcription was performed using PrimeScript RT reagent kit (TAKARA BIO INC., Japan) starting from 1 µg of RNA-DNA free template. Real-time PCR amplification was performed in LightCycler 480 System (Roche Molecular Systems, Inc., Switzerland), using 5 µl of NZYSupreme qPCR Green Master Mix (2x) (NZYTech, Lisboa, Portugal), 0.5 µl of forward and reverse primers (10 mM) and 2µl of cDNA, resulting in a total reaction volume of 10 µl. The reaction was performed using the following PCR conditions: 1 cycle at 95°C for 2 minutes, 40 cycles at 95°C for 15 seconds, 58°C for 30 seconds, 72°C for 30 seconds, and a final melting cycle at 95°C for 5 seconds and 65°C for 1 minute. The relative fold gene expression of the samples was calculated using the $2^{\Delta\Delta CT}$ method described by Ricupero et al. (2023).

Table 1. Primer sequences for target and housekeeping genes used in the transcriptional analysis of *Ceratitis capitata* detoxification pathways.

Gene	Gene description	Primer sequence (5'-3')
TUB1a (HK)	Tubulin Alpha	FW: ACATTACTATCCGGCTACACAG
		RV: TGATAATTCTTCGAGCGTCCAC
AANAT	Aralkylamine N-Acetyltransferase	FW: GTCGGATTCATGCCAACACC
		RV: GCTATGCCCAGTCCACGAT
AChE	Acetylcholinesterase	FW: TTTGCAGGCACCCTCGTC
		RV: ACCACGACCGATGTCAC
Adh	Alcohol Dehydrogenase	FW: AATGAATCGGCTTACAACGAAA
		RV: TTCCTCACATATGCCAACACC
GABAT	Gamma-Aminobutyric Acid Transaminase	FW: AAGAACTTTAGTCAACCGTCCT
		RV: TTCGAACACGATCCGCACA
GSTS1	Glutathione S Transferase S1	FW: TTCGATGGCCCGCTTT
		RV: ATCTCATCCTCCGGTTCGT
MGSTL1	Microsomal Glutathione S-Transferase 1	FW: GGATTTATTGGACAAACGCCTGAA
		RV: CACATAAAGGAAACCAATGACGAAG
TBH	Tyramine Beta Hydroxylase	FW: CACCACAGCCATTGCCGAG
		RV: AGCCAAATCTTCACGAGCTT
Cyp6g1	Cytochrome P450 6g1	FW: CGAACACCTTTGACCCGGAA
		RV: GCACAAGTCGCAACATAGTGA
Cyp6g2	Cytochrome P450 6g2	FW: CGCCAGCCTCTTCCGA
		RV: CGTAAATGACGCCACTAGCA
Cyp6a2	Cytochrome P450 6a2	FW: ACCGGAACGCCTCGAC
		RV: AGCGCAGACCTATGCAA
Cyp6a69	Cytochrome P450 6a69	FW: TTGCGCCTTCGGACTCGAG
		RV: TAGAAATTGTGCAGCGGTTGTGGT
Cyp6t3	Cytochrome P450 6t3	FW: ATCGCGTTGATTTTATACGAGCTG
		RV: GCCTTCCGCAACCACC
Cyp12e1	Cytochrome P450 12e1	FW: CCGGCACAGACATATCCATC
		RV: TCATTACGCAGCCAACGTTC

2.6 Effects of garlic-NE on *C. capitata* microbiome

The influence of the garlic-NE treatment on the bacterial communities of *C. capitata* adults was evaluated using the above-mentioned ingestion trials (see section 2.4.1). A total of three treatments were carried out using the estimated LD_{30} (0.69 % of EO) (see Table 3), sucrose water solution (30% w/v) (C-), and a sucrose/water/tween solution (30/65/5 % w/v/w) (Ct). Four replicates were performed for each treatment with a pool of three medflies each. In detail, for every

treatment, the specimens were fed daily with four drops (50 µL each) until the end of the experiment (72 hours). After 24 and 72 hours, the insects were placed into Eppendorf tubes (1.5 mL), immediately ground in liquid nitrogen, and stored at -80°C until use. Firstly, the samples were immersed in sodium hypochlorite solution (0.1%) and then in sterile water to sterilize the insect's surfaces. Total DNA was extracted using the NZY Tissue Gdna isolation kit (Nzytech) kit following the manufacturer's protocol. Bacterial communities were characterized through the amplification of the hypervariable region V3 and V4 using primers 341F (CCTACGGGNGGCWGCAG) and 805R (GACTACHVGGGTATCTAATC). Amplicon library preparation was performed according to recommended protocols (Illumina Demonstrated Protocol: 16S Metagenomic Sequencing Library Preparation) and then sequenced on an Illumina Miseq by Macrogen (Netherlands).

2.7 Data analysis

Statistical analyses were performed using IBM® SPSS® Statistics v. 23 (IBM Corp. Released 2015. Armonk, NY, USA). All data met the assumptions required by parametric tests, including normality and homoscedasticity of variance ($P > 0.05$). Differences among the developed EO-NEs (i.e., garlic, eucalyptus, and clove-NE) in the physical characteristics were subjected to analysis of variance (ANOVA) with size, PDI, and surface charge as dependent variables, and EO-NEs used as fixed factors. *Ceratitis capitata* mortality data were corrected for control mortality using Abbott's formula (Abbott, 1925). The ANOVA test was used to assess the difference among the treatments in the preliminary test, the serial dilutions trial against *C. capitata,* and the toxicity bioassay towards a natural antagonist such as *A. daci*. The data obtained in the serial dilution bioassay were subjected to a probit analysis to estimate the LD_{50} and LD_{90} values and their 95% fiducial limits. The statistical differences among the treatments (i.e. C-, $LD_{50,}$ and LD_{90}) on the transcription level of *C. capitata* genes were subjected to ANOVA analysis.

The raw 16S rRNA gene amplicon data were analyzed in RStudio v4.2.2 using the DADA2 R package (Callahan et al., 2016). Read-quality profiles were inspected, and low-quality reads were subsequently filtered and deleted. The selected sequences were merged, dereplicated, and classified into unique Amplicon Sequence Variants (ASVs). ASVs with 100% sequence identity were preferred over operational taxonomic units (OTU) due to their superior reproducibility and resolution (Callahan et al., 2017). The ASVs identified as mitochondria or chloroplasts were removed from the dataset. Taxonomic classification of the ASVs was performed using a classify-by-consensus approach, with Silva SSU (v. 138.1) as the reference database (Quast et al., 2012), and

normalized using the Total Sum Scaling (TSS) method. The MicrobiotaProcess package (Xu et al., 2023) was used to perform alpha- and beta-diversity analyses and to identify the key taxa comprising the core microbiome. Alpha diversity, which assesses within-sample diversity, was evaluated using the Chao1, Shannon, and Simpson 1-D indices. Beta diversity was evaluated using analysis of similarities (ANOSIM) (Anderson and Walsh, 2013) and permutational multivariate analysis of variance (PERMANOVA) (Anderson, 2001), using 999 permutations with the "adonis" function. The dispersion among the groups was evaluated by multivariate homogeneity of group dispersion test (PERMDISP) (Anderson, 2006), using the "betadisper" function. All analyses were carried out using the vegan package (Oksanen et al. 2022). To underline shared ASVs among the groups, Venn diagrams were made using ggplot2 R-package (Wickham 2016). The sequencing data are available in the NCBI database under project number PRJNA1214852.

3. RESULTS

3.1 Physical characterization of developed EO-NEs

The size and zeta potential distribution of the developed EO-based nano-emulsions, such as garlic-NE, eucalyptus-NE, and clove-NE, are shown in Supp. Fig. 1,2, and 3, respectively. The analysis of their physical characteristics confirmed that all EO-NEs exhibited particle size within the nanoscale range (< 200 nm), with low polydispersity index (PDI) values (<0.2) and negative surface charge, indicating good stability due to repulsion among particles. Notably, garlic-NE and eucalyptus-NE had the smallest particle sizes and the lowest PDI values, while clove-NE showed comparatively larger particle sizes and higher PDI. Regarding surface charge, clove-NE exhibited the lowest zeta potential among the tested nano-emulsions. Statistical differences were observed among all EO-NEs for the analyzed physical characteristics ($P < 0.05$) (Table 2).

Table 2: Physical characteristics (size, PDI, and zeta potential) of developed EO-NEs 24h after development. Values are the mean (± standard deviation) of three replicates. Different letters indicate statistical differences among the EO-NEs within the same physical properties ($P < 0.05$; ANOVA).

EO-NE[1]	Size (nm)	PDI[2]	Zeta Potential (mV)
Garlic-NE	61.7 ± 0.28 a	0.049 ± 0.003 a	-11.1 ± 0.21 a
Eucalyptus-NE	63.86 ± 0.34 a	0.088 ± 0.013 b	-11.0 ± 0.46 a
Clove-NE	179.9 ± 2.21 b	0.175 ± 0.016 c	-23.4 ± 0.05 b
(*F;* df; *P level*)	(8065.05; 2; <*0.001*)	(87.31; 2; <*0.001*)	(103.19; 2; <*0.001*)

[1]Essential oil-based nano-emulsion; [2]Polydispersity index.

3.2 Insecticidal activity of developed EO-NEs

The initial bioassay using a high concentration of active ingredient (2.5% EO) revealed significant differences in the toxicity of the developed EO-NEs against *C. capitata* adults. Garlic-NE exhibited the highest efficacy, reaching nearly 100% mortality at 72 hours post-exposure. In contrast, clove-NE induced an intermediate mortality rate, while eucalyptus-NE showed the lowest toxicity throughout the experiment. Statistical analysis confirmed significant differences among treatments 48 and 72 hours after the exposure ($F = 21.95$; df= 2; $P < 0.001$ and $F = 38.02$; df= 2; $P < 0.001$, respectively), with garlic-NE consistently outperforming the other formulations (Fig. 1).

The dose-response bioassay confirmed a significant positive correlation between garlic-NE concentration and *C. capitata* adult mortality at 72 hours post-exposure (Fig. 2). The experimental

data highlighted a dose-dependent mortality, with statistical differences observed among the tested doses (F = 26.1; df = 1, 4; P < 0.01). Mortality increased progressively with higher EO concentrations, reaching nearly 90% at the highest tested dose (1.87% EO). Mortality rates at 0.46% and 0.625% EO remained low and statistically similar, whereas doses of 0.93% EO and above significantly increased insect mortality. Furthermore, the experimental data fitted with the Probit model (X^2 = 7.438; df = 1, 3; P = 0.19), and the lethal doses and their fiducial limits were estimated (Table 3).

The toxicity of garlic-NE against *A. daci* adults at 72 hours post-exposure is presented in **Figure 3**. The results demonstrated a highly lethal effect, with 100% mortality observed for both tested doses (LD_{50} = 0.87% EO and LD_{90} = 1.91% EO). No significant differences were detected between the lethal doses and the positive control (C+; Spintor™ Fly). No mortality was recorded in the negative control (C-; sucrose water solution 30% w/v).

Figure 1. Mortality % (mean ± SE) of *C. capitata* adults at 24, 48 and 72 hours after exposure to garlic, eucalyptus, and clove NEs (2.5% of EO). Different letters indicate significant differences among treatments within the same exposure time (P < 0.05).

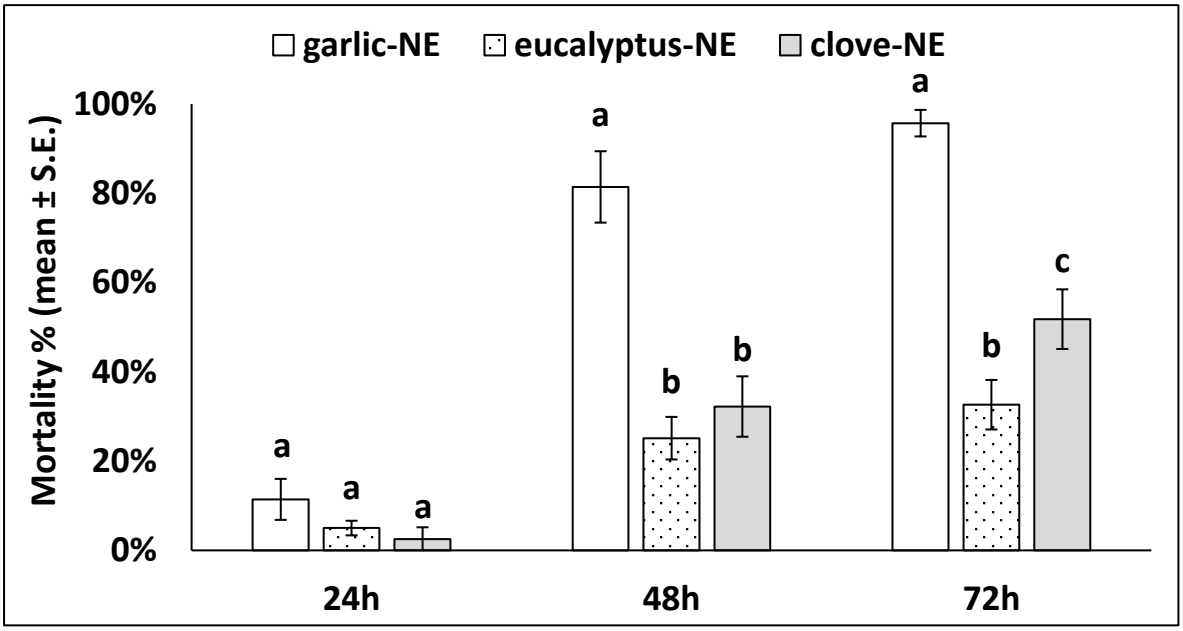


Figure 2. Dose-response mortality (mean ± SE) of garlic-NE dilutions (% of EO) against *C. capitata* adults 72 hours after exposure. Different letters indicate statistical differences among the doses ($P < 0.05$).

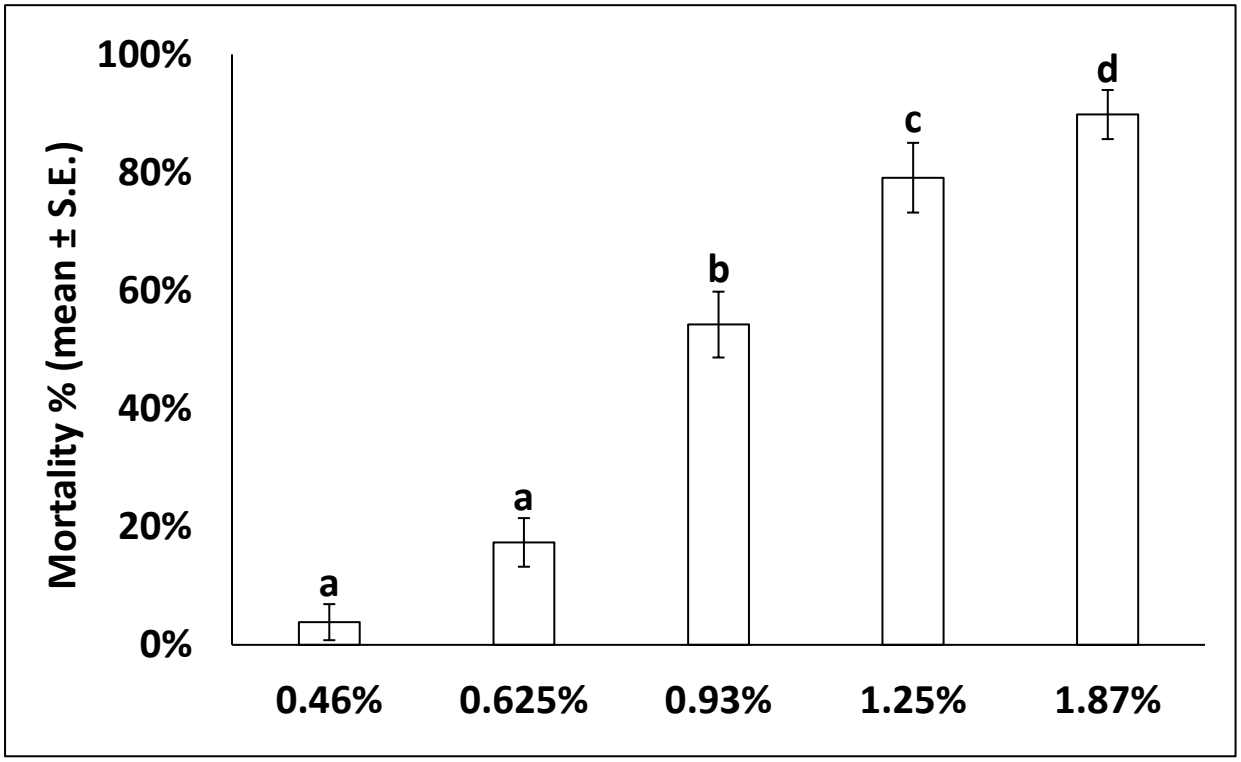
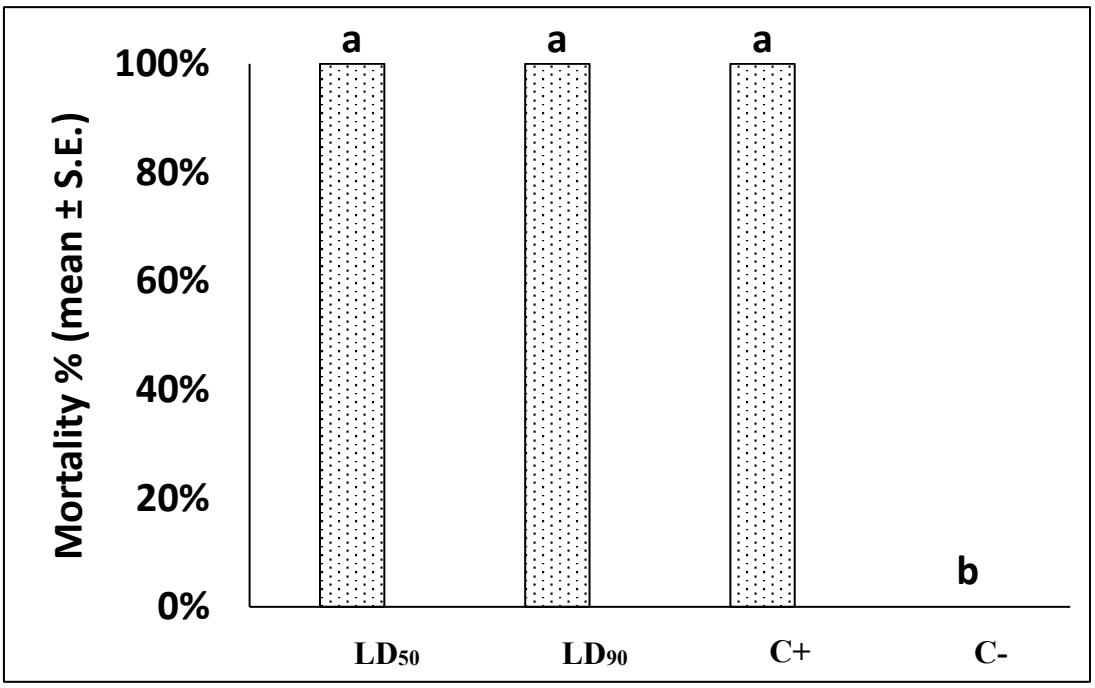


Table 3. Estimated lethal doses (LD_{30}, LD_{50}, and LD_{90}) of garlic-NE against *C. capitata* adults. Values were considered statistically different if their 95% fiducial limits did not overlap.

LD[1]	Estimate	95% Confidence Limits garlic-NE doses %		*X* (df[2])	*P* level
		Lower bound	Upper bound		
LD_{30}	0.69	0.61	0.76		
LD_{50}	0.96	0.87	1.06	7.438 (3)	0.19
LD_{90}	2.18	1.91	2.55		

[1] Lethal dose; [2] Degrees of freedom.

Figure 3. Mortality % (mean ± SE) of *Aganaspis daci* adults 72 hours after exposure to garlic-NE at LD_{50}, LD_{90}, positive control (C+; Spintor™ Fly), and negative control (C-; sucrose water solution 30% w/v). Different letters indicate significant differences among treatments ($P < 0.05$).



3.3 Impact of garlic-NE on gene expression against *C. capitata*

The study of transcriptional levels of the genes *AANAT*, *AChE*, *AdH*, *GABAT*, *GSTS1*, *MGSTL1*, *TBH*, *Cyp6g1*, *Cyp6g2*, *Cyp6a2*, *Cyp6a69*, *Cyp6t3*, and *Cyp12e1* highlighted statistical differences among the different treatments (i.e., C-, LD_{50}, and LD_{90}), particularly in the genes *Adh*, *GSTS1*, *Cyp6g2*, *Cyp6a2*, and *Cyp6a69* ($P < 0.05$) (Table 4). The *Adh* gene, involved in Phase I detoxification and alcohol metabolism, exhibited a dose-dependent response, with significant differences among treatments. The glutathione S-transferase gene (*GSTS1*) showed a significant overexpression only at the highest concentration (LD_{90}), indicating an activation of Phase II detoxification. In contrast, the Cytochrome *P450* gene (*Cyp6g2*), involved in the oxidative metabolism of xenobiotics, was significantly upregulated at the LD_{50} dose. At the same time, *Cyp6a2* and *Cyp6a69*, also part of the Phase I detoxification, were overexpressed at both tested doses compared to the control. Notably, the increase in *Cyp6a69* expression at LD_{50} and LD_{90} was nearly twice that of the control. Statistical differences among treatments for all genes analyzed are summarized in Table 4.

Table 4. Relative expression HK/gene (mean ± standard error) of different *C. capitata* genes 24 hours after the treatment with C- (sucrose-water solution 30% w/v), LD_{50} (0.96% of EO), and LD_{90} (2.18% of EO) of garlic-NE. Transcriptional levels were normalized to the expression of housekeeping (*TUB1a*). Different letters indicate statistical differences among the treatments within the same gene ($P < 0.05$).

Gene	Treatment	Relative expression (± S.E.)	F (df^1=2)	P
Adh	C-	0.66 ± 0.05 a	38.06	*<0.001*
	LD_{50}	1.15 ± 0.04 b		
	LD_{90}	1.32 ± 0.09 c		
GSTS1	C-	1.13 ± 0.07 a	6.62	*0.005*
	LD_{50}	1.57 ± 0.11 ab		
	LD_{90}	1.61 ± 0.08 b		
Cyp6g2	C-	1.12 ± 0.16 a	5.31	*0.01*
	LD_{50}	1.90 ± 0.22 b		
	LD_{90}	1.21 ± 0.16 a		
Cyp6a2	C-	0.85 ± 0.12 a	7.62	*0.002*
	LD_{50}	1.47 ± 0.15 b		
	LD_{90}	1.49 ± 0.12 b		
Cyp6a69	C-	0.83 ± 0.27 a	3.36)	*0.014*
	LD_{50}	1.77 ± 0.21 b		
	LD_{90}	1.95 ± 0.37 b		

[1]Degrees of freedom.

3.4 Effects of garlic-NE on *C. capitata* microbiome

The bacterial community composition of *C. capitata* was analyzed at 24 and 72 hours post-treatment. Across all conditions, *Enterobacteriaceae*, particularly the genus *Pluralibacter*, dominated the microbiota. When *Pluralibacter* spp. was excluded, treatment-related differences became more apparent (Fig. 4).

At 24 hours, all groups (C-, LD_{30}, Ct) were predominantly composed of *Erwiniaceae*, but secondary bacterial families varied. LD_{30} showed a higher relative abundance of *Sphingomonadaceae* and *Halomonadaceae*, *C-* exhibited *Enterococcaceae* and *Pseudomonadaceae*, while *Ct* was enriched in *Rhizobiaceae* and *Halomonadaceae*. By 72 hours, *Erwiniaceae* remained dominant, but microbial composition changed: LD_{30} showed an increased proportion of *Staphylococcaceae* and

Moraxellaceae, *C-* had a higher presence of *Enterococcaceae*, and *Ct* displayed enrichment in *Halomonadaceae* and *Burkholderiaceae* (Fig. 4).

Despite these compositional variations, alpha diversity indices (Chao1, Shannon, and Simpson) did not show significant differences among treatments at either time point ($P > 0.05$, Fig. 5). Similarly, beta diversity analysis revealed no significant differences at 24 hours. However, at 72 hours, the microbial community structure differed among treatments, as indicated by PERMANOVA ($P = 0.05$) and ANOSIM ($P = 0.016$). PCA and PCoA visualizations further supported this finding, showing distinct clustering patterns at 72 hours (Fig. 6).

Temporal analysis within each treatment showed a decline in shared amplicon sequence variants (ASVs) over time. At 24 hours, 19% of ASVs (n = 16) were shared across treatments, with *C-* (20%), *Ct* (32%), and LD_{30} (18%) having similar proportions of unique ASVs. At 72 hours, the proportion of shared ASVs remained stable (19%), but their absolute number was reduced by half (n = 8). Notably, LD_{30} retained a higher proportion of unique ASVs (37%) compared to C- (16%) and Ct (9.3%) (Fig. 6). Beta diversity analysis across the entire dataset showed no significant differences. However, when *Pluralibacter* spp. was excluded, a significant interaction between treatment and time was detected (PERMANOVA $P < 0.05$; ANOSIM $P < 0.05$). This suggests garlic-NE induces gradual shifts in the *C. capitata* microbiome, with more pronounced effects at 72 hours post-treatment.

Figure 4. Bacterial community composition of *Ceratitis capitata* after garlic-NE treatment: Relative abundance of bacterial families at 24 and 72 hours post-treatment. Treatments: C- (sucrose/water solution 30% w/v), LD_{30} (0.69% EO), and Ct (sucrose/water/Tween 80 solution 30/65/5% w/v/w).

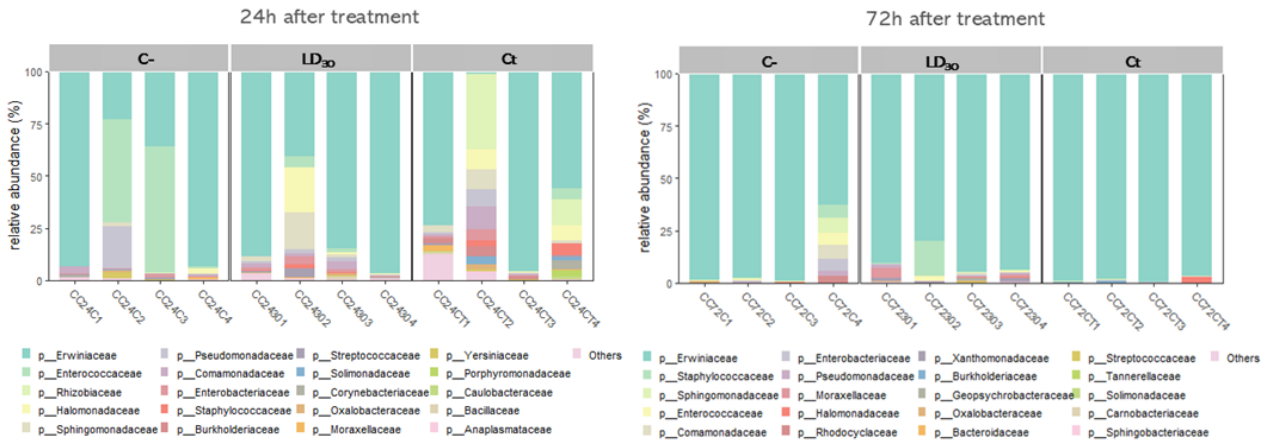


Figure 5. Different alpha diversity indexes (i.e. Chao1, Shannon, and Simpson) after 24h (A) and 72h (B), in *C. capitata* treated and untreated samples (i.e. C-, LD_{30}, and Ct).

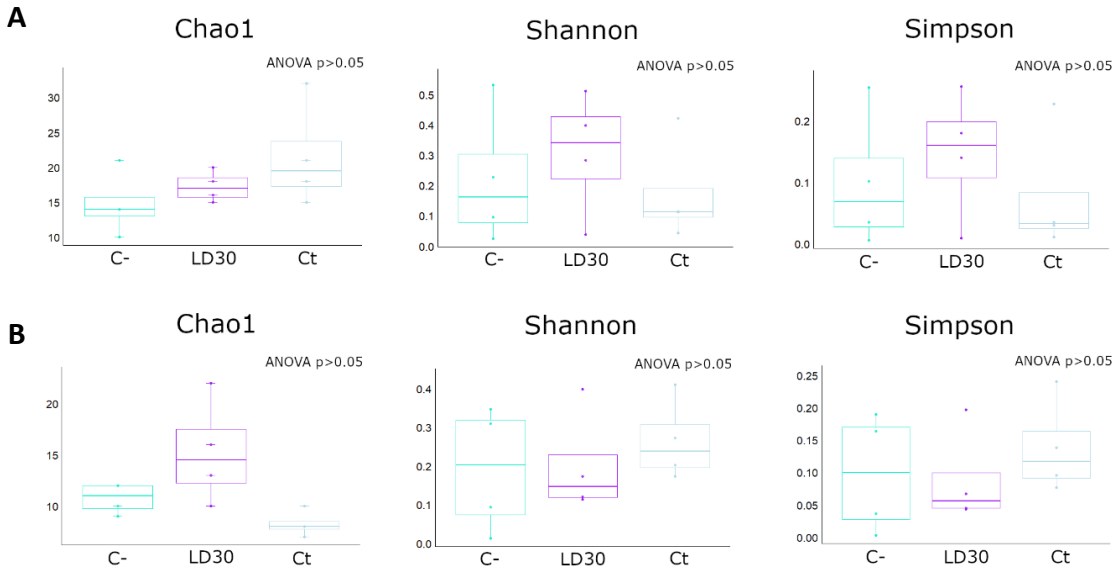
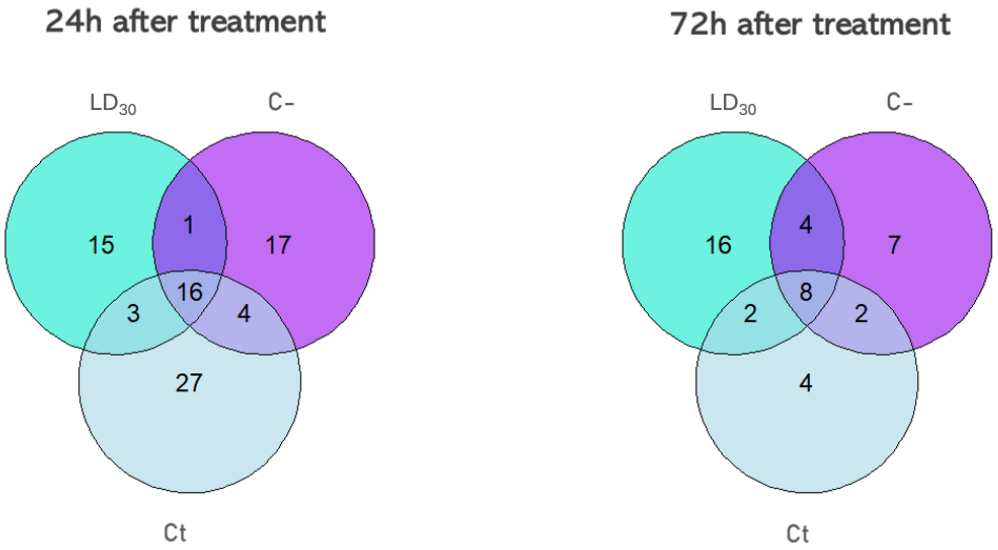


Figure 6. Venn diagrams showing the number of shared and unique taxa among *Ceratitis capitata* treated and untreated samples (i.e. LD_{30}, C-, and Ct) after 24h and 72h, at the genus taxonomic level.



4. DISCUSSION

To our knowledge, this study highlighted the toxic effects of EO-based nano-emulsions against *C. capitata* adults for the first time. Generally, the biological activities of these nano-formulations are influenced by several factors, including the plant species, the amount of EO, the application rate, and the physical properties (particle size, PDI, and surface charges) of the nano-formulations. Among these, the physical properties, particularly the particle size, play a key role in toxic mechanisms against pests. Indeed, as reported by Modafferi et al. (2024b), only the nano-formulations that have particle sizes ranging in the nanoscale (<250 nm) can particularly affect the survivability of the exposed insects, unlike the one that did not have nano-particle size. Several authors reported the association between nanoparticle size and good insecticidal activity in this context. For instance, Giuliano et al. (2024) developed a garlic EO-based nano-emulsion and investigated its biological activity against *Spodoptera littoralis* Boisduval (Lepidoptera: Noctuidae). Despite the garlic-NE exhibited particle sizes (141.0 ± 1.375 nm) more than double compared with that obtained in this study (61.7 ± 0.28), showed high insecticidal activity with estimated LD_{50} and LD_{90} of 1.57 and 2.39 % of EO respectively. Similarly, Hassan et al. (2023) developed a clove NE with a particle size (158.4 ± 0.353) similar to that we obtained (179.9 ± 2.21) and showed good insecticidal activity against adult fleas with an estimated LD_{50} and LD_{90} of 26.42 and 49.16 µg/ml, respectively. Other authors, developed eucalyptus EO-based nano-emulsions with droplet sizes (< 10 nm) less than the results obtained by our eucalyptus-NE (63.86 ± 0.34) and highlighted a good insecticidal activity against *Pectinophora gossypiella* (Saunders) (Lepidoptera: Gelechiidae), *Culex quinquefasciatus* Say (Diptera: Culicidae), *Sitophilus oryzae* (Linnaeus, 1764) (Coleoptera: Curculionidae), *Rhyzopertha dominica* (Fabricius, 1792) (Coleoptera: Bostrichidae), and *Tribolium castaneum* (Herbst.) (Coleoptera: Tenebrionidae) (Mohammed and Nasr, 2020; Moustafa et al., 2015; Sugumar et al., 2014).

Despite the excellent physical characteristics exhibited by all EO-NEs, only garlic-NE exhibited high insecticidal activity (more than 90% of mortality) against the target pest in the preliminary trials. Conversely, clove-NE and eucalyptus-NE showed an intermediate and low mortality rate. As far as we know, few studies have investigated the efficacy of these nano-formulations against pests belonging to the Tephritidae family. Giunti et al. (2022a) evaluated the biological activity of three EO-NEs (anise, fennel, and mint-NE) against *Bactrocera oleae* (Rossi) (Diptera: Tephritidae). The results showed the ovideterrent activity of these nano-formulations, although the same formulations did not affect the insect survival in contact toxicity trials. In contrast, our garlic-NE,

despite a different route of exposure (ingestion toxicity), showed good insecticidal activity against *C. capitata*, highlighting a dose-response mortality rate with estimated LD_{50} and LD_{90} of 0.96 and 2.18% of EO, respectively. Although the efficacy of these EO-based nano-delivery systems against tephritid pests was poorly documented, the toxicity of EOs, used as such, was well-investigated against this family pest. Indeed, several authors reported the biological activity of different EOs against *C. capitata* (Benelli et al., 2013; Miguel et al., 2010; Passino et al., 1999). For instance, Medflies fed with 5% of *Thymus herba-barona* and *Cinnamomum zeylanicum* EOs showed a mortality rate of more than >90%, whereas *Salvia officinalis* and *Rosmarinus officinalis* EOs induced a low insect mortality (<50%) (Moretti et al., 1998). In contrast, other studies tested the efficacy of different EOs, including *R. officinalis*, with a mortality rate of 100% against Medflies fed with diets containing 2.5% of EOs (Benelli et al., 2012). Similarly, EOs extracted from *Eucalyptus campaspe* and *Eucalyptus torquata* were effective in terms of toxicity and repellence activity against C. capitata adults through different routes of exposure (ingestion, contact, and fumigation application) (Nenaah et al. 2015).

One of the most interesting aspects related to the use of the botanicals (i.e., EOs) used as such or encapsulated into nano-delivery systems is their presumed safety toward non-target. Modafferi et al. (2024a) discussed that the biological activity of these nano-formulations toward non-target organisms was poorly investigated. Knowledge about the harmful effects must be implemented considering different factors such as type of natural substances and nano-formulation, application rate, doses, insect species or route of exposure (Giunti et al., 2022b). Several studies have explored the use of garlic essential oil-based nano-emulsions (EO-NEs), assessing their impact on non-target organisms. For instance, Modafferi et al. (2024b, 2024a) and Ricupero et al. (2022) investigated effects of garlic-NEs on non-target coccinellid predators, such as *Cryptolaemus montrouzieri* (Mulsant) (Coleoptera: Coccinellidae), as well as honeybees and tomato plants reporting no significant adverse effects. Similarly, Papanikolaou et al. (2018) showed that a natural pyrethrin-based nano-emulsion was effective against *Aphis gossypii* (Glover) (Hemiptera: Aphididae), whereas it did not show toxicity towards two aphid predators, *Coccinella septempunctata* L. (Coleoptera: Coccinellidae) and *Macrolophus pygmaeus* Rambur (Hemiptera: Miridae). Although several authors did not report adverse effects of these substances against non-target insects, the results in the literature are in contrast. Indeed, some of these new nano-formulations can affect non-target organisms. Giuliano et al. (2024) reported that the pepper plants treated with garlic-NE showed low phytotoxic effects on the leaves and reduced fruit production compared to untreated

plants. Similarly, a mandarin-NE exhibited lethal and sub-lethal effects toward *Nesidiocoris tenuis* Reuter (Hemiptera: Miridae) (Campolo et al., 2020). According to these, our results showed that the developed garlic-NE was extremely toxic towards *A. daci* and both the tested lethal doses (LD_{50} of 0.96% of EO and LD_{90} of 2.18% of EO) led to the death of all the wasp specimens. These results suggest that garlic-NE should be used carefully with biological control programs. However, it is important to consider that treatments against *C. capitata* are typically carried out using bait formulations (Giunti et al., 2023b), which are significantly more selective toward natural enemies (Urbaneja et al., 2009). As tested in this study, garlic-NE was also applied in bait treatments, highlighting the need to evaluate their selectivity towards natural enemies in future research.

Essential oils (EOs) are known to interfere with the insect nervous system through multiple mechanisms, including modulation of octopamine and GABA receptors, alteration of calcium ion channels, and inhibition of acetylcholinesterase (AChE) and ATPase activity (Campolo et al., 2018; Enan, 2001; Kostyukovsky et al., 2002; Wang and Heinbockel, 2018). However, these effects vary depending on EO composition and concentration. For instance, Park et al. (2016) demonstrated that certain EO compounds, such as perilla aldehyde and thymol, can inhibit AChE activity by approximately 60%.

In contrast, our results did not reveal significant changes in the expression of genes involved in neurotransmitter metabolism and synaptic regulation (*AChE, TBH, GABAT*) among treatments (C-, LD_{50}, and LD_{90}). This suggests that garlic-NE does not exert its toxicity through direct neurotoxic effects, distinguishing it from conventional insecticides that target the nervous system. Instead, transcriptional analyses indicated a significant upregulation of genes involved in Phase I (cytochrome P450 monooxygenases) and Phase II (glutathione S-transferases) detoxification pathways, pointing to a metabolic detoxification response as the primary mode of action.

Among Phase I detoxification genes, *Cyp6a2, Cyp6a69,* and *Cyp6g2* were significantly upregulated, with *Cyp6a69* showing nearly twice the expression levels of the control at LD_{50} and LD_{90}. These genes are widely associated with xenobiotic metabolism and insecticide resistance, playing a key role in oxidative detoxification and metabolic adaptation to toxic compounds (Akami et al., 2019). Similarly, the increased expression of *Adh* suggests a metabolic response to volatile alcohol-derived compounds present in garlic-NE. Regarding Phase II detoxification, *GSTS1* was significantly overexpressed at LD_{90}, indicating that glutathione conjugation plays a role in neutralizing and excreting garlic-NE-derived metabolites. This aligns with previous studies showing the induction of glutathione S-transferases (GSTs) in insects exposed to plant-based

bioinsecticides. For example, Gao et al. (2020) reported the overexpression of multiple GST genes (*Gsts4, Gsts6, and Gsts7*) in *Tribolium castaneum* larvae fed with *Artemisia vulgaris* EO, supporting the hypothesis that GST-mediated detoxification is a conserved response to plant-derived toxins. These results highlight a fundamental difference between the mode of action of EO-based bioinsecticides and conventional neurotoxic insecticides. While organophosphates and carbamates primarily inhibit AChE activity (Abdelgaleil et al., 2009; Kostyukovsky et al., 2002) and neonicotinoids target nicotinic acetylcholine receptors (Casida, 2018), garlic-NE induces a broader metabolic detoxification response. This suggests that its insecticidal activity may stem from overloading the metabolic detoxification system, leading to the accumulation of toxic intermediates and oxidative stress, rather than a direct neurotoxic effect. Moreover, this multi-pathway activation of detoxification genes could significantly impact resistance management. Conventional insecticides often target a single metabolic or neural pathway, facilitating the development of resistance in pest populations. In contrast, garlic-NE requires insects to activate multiple metabolic pathways simultaneously to process its diverse blend of bioactive compounds. This complex mode of action reduces the likelihood of resistance development, supporting the potential of essential oils as sustainable alternatives for integrated pest management (IPM) strategies.

The insect gut microbiome plays a fundamental role in various physiological processes, including digestion, immune system regulation, and detoxification of xenobiotics, which can influence pesticide resistance (Engel & Moran, 2013; Douglas, 2015). Several studies have demonstrated the involvement of gut microbiota in insecticide metabolism, as specific bacterial taxa contribute to the degradation and detoxification of toxic compounds (Kikuchi et al., 2012; Xia et al., 2018). Specifically, members of the Enterobacteriaceae family have been linked to insecticide resistance through their ability to metabolize xenobiotics, as reported in *Bactrocera dorsalis* (Cheng et al., 2017).

In agreement with previous studies, our results confirmed that Enterobacteriaceae represents a dominant taxon in the gut microbiome of *C. capitata* (Malacrinò et al., 2018; Mason et al., 2023), with *Pluralibacter* spp. being the most abundant genus across all treatments. However, after excluding *Pluralibacter* from the analysis, apparent shifts in bacterial composition were observed among treatments, suggesting that garlic-NE selectively influences the microbiome structure. At 24 hours post-treatment, the LD_{30} group's microbial composition was characterized by an increased prevalence of Erwiniaceae, followed by Sphingomonadaceae and Halomonadaceae,

whereas the control groups exhibited distinct microbial signatures. Specifically, Enterococcaceae and Pseudomonadaceae were predominant in the C- group, while Rhizobiaceae and Halomonadaceae were more abundant in the Ct group. Since certain *Enterobacteriaceae*, such as *Pluralibacter spp.*, have been linked to insecticide degradation and resistance mechanisms (Cheng et al., 2017), their altered representation in the garlic-NE-treated group may influence the detoxification capacity of *C. capitata*. However, further functional analyses are needed to determine whether these shifts translate into impaired xenobiotic metabolism. This suggests that garlic-NE induces an early microbial shift, likely due to its antimicrobial activity, which selectively affects bacterial taxa. At 72 hours post-treatment, significant beta diversity changes were detected, indicating a more pronounced restructuring of the gut microbiota. When *Pluralibacter spp.* was excluded, microbial composition in the LD_{30} group showed a marked increase in *Staphylococcaceae* and *Moraxellaceae*. In contrast, the C- and Ct groups maintained a higher proportion of *Enterococcaceae* and *Halomonadaceae*. Interestingly, the increase in *Staphylococcaceae* and *Moraxellaceae* in the garlic-NE-treated group suggests a shift in microbial dominance, potentially affecting the functional contributions of gut bacteria to detoxification. The lower presence of *Pseudomonadaceae* and *Enterococcaceae* in treated groups could indicate a disruption in bacterial-mediated metabolism of toxic compounds. These findings support the hypothesis that garlic-NE exerts a progressive antimicrobial effect, disrupting microbial homeostasis over time.

The antimicrobial properties of essential oils (EOs) and their nanoemulsions against insect-associated bacteria have been well documented (Dehghankar et al., 2021; Szczepanik et al., 2018). The observed shifts in microbial community structure suggest that garlic-NE selectively modulates gut bacterial populations, potentially altering microbiome functionality. Given the role of certain gut bacteria in xenobiotic metabolism, these microbial changes may compromise the insect's ability to detoxify garlic-NE components. The loss of key microbial taxa could weaken the insect's overall physiological resilience, increasing its susceptibility to the treatment.

Unlike conventional neurotoxic insecticides, which act directly on the nervous system, garlic-NE triggers metabolic stress, activating detoxification pathway and significant microbiome alterations. Since gut bacteria contribute to xenobiotic metabolism and nutrient assimilation, disrupting this balance may impair the insect's detoxification capacity and compromise physiological homeostasis. This dual impact—metabolic disruption combined with microbiome

restructuring—suggests a multifaceted mode of action, potentially reducing the likelihood of resistance development by targeting multiple physiological pathways simultaneously.

Overall, our results indicate that garlic-NE disrupts the gut microbiome of *C. capitata*, with microbial shifts becoming more pronounced over time. This restructuring may contribute to the insecticidal effects of garlic-NE, either by interfering with microbial-mediated detoxification processes or by inducing gut dysbiosis, which could impact host fitness and survival. Disrupting microbial homeostasis could profoundly affect insect physiology, particularly in metabolic processes associated with xenobiotic degradation. While our study demonstrates that garlic-NE induces structural changes in the microbiota, future research should assess the functional consequences of these shifts, particularly their impact on enzymatic pathways related to insecticide detoxification.

5. CONCLUSION

This study highlights the potential of essential oil-based nano-emulsions (EO-NEs) as innovative tools for pest management, offering an alternative to synthetic insecticides. The successful application of microfluidization demonstrates its effectiveness in enhancing EO stability, overcoming key limitations such as high volatility and poor solubility. The findings suggest that garlic-NE exerts its insecticidal action through metabolic disruption rather than direct neurotoxicity, activating multiple detoxification pathways and altering the gut microbiota of *C. capitata*. This multifaceted mode of action could reduce the likelihood of resistance development, a major concern in pest control. Our findings indicate that garlic-NE toxicity in *C. capitata* is not solely attributed to its direct insecticidal effects but also to its ability to disrupt key metabolic pathways and alter the gut microbiota. These microbiome shifts may impair detoxification efficiency, increasing susceptibility to toxic stressors. Understanding this complex interaction between metabolic detoxification and microbiota modulation provides new insights into the mode of action of essential oil-based nanoemulsions and highlights their potential as sustainable alternatives for pest management. However, the impact of garlic-NE on non-target organisms, particularly beneficial insects, raises concerns about its selectivity and suitability for Integrated Pest Management (IPM) strategies. Future research should minimize off-target effects, evaluate long-term ecological consequences, and optimize its use in real-world agricultural settings. The development of EO-based nanoformulations represents a promising step toward more sustainable pest control solutions. Still, their integration into commercial applications requires further refinement to balance efficacy, environmental safety, and selectivity.

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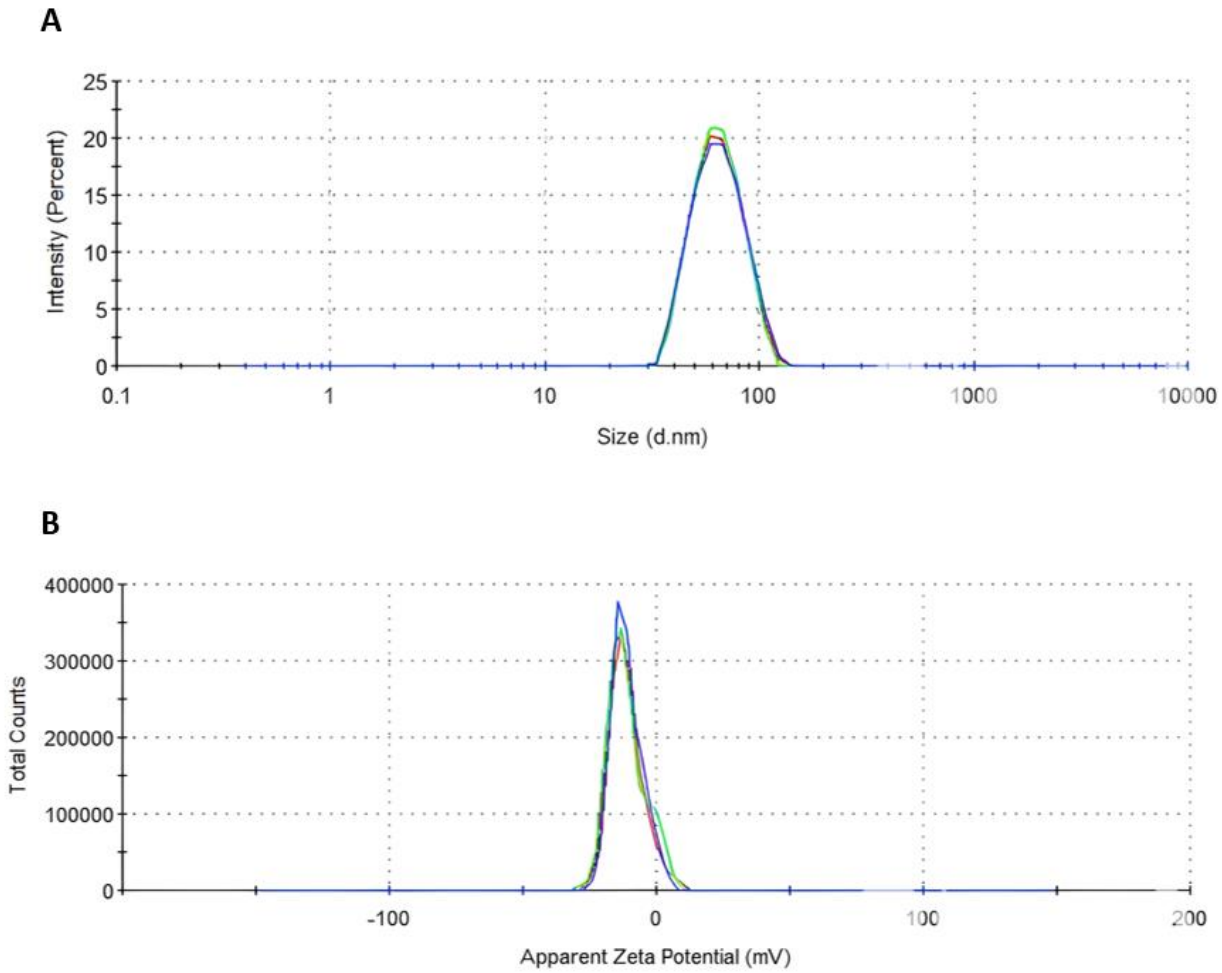
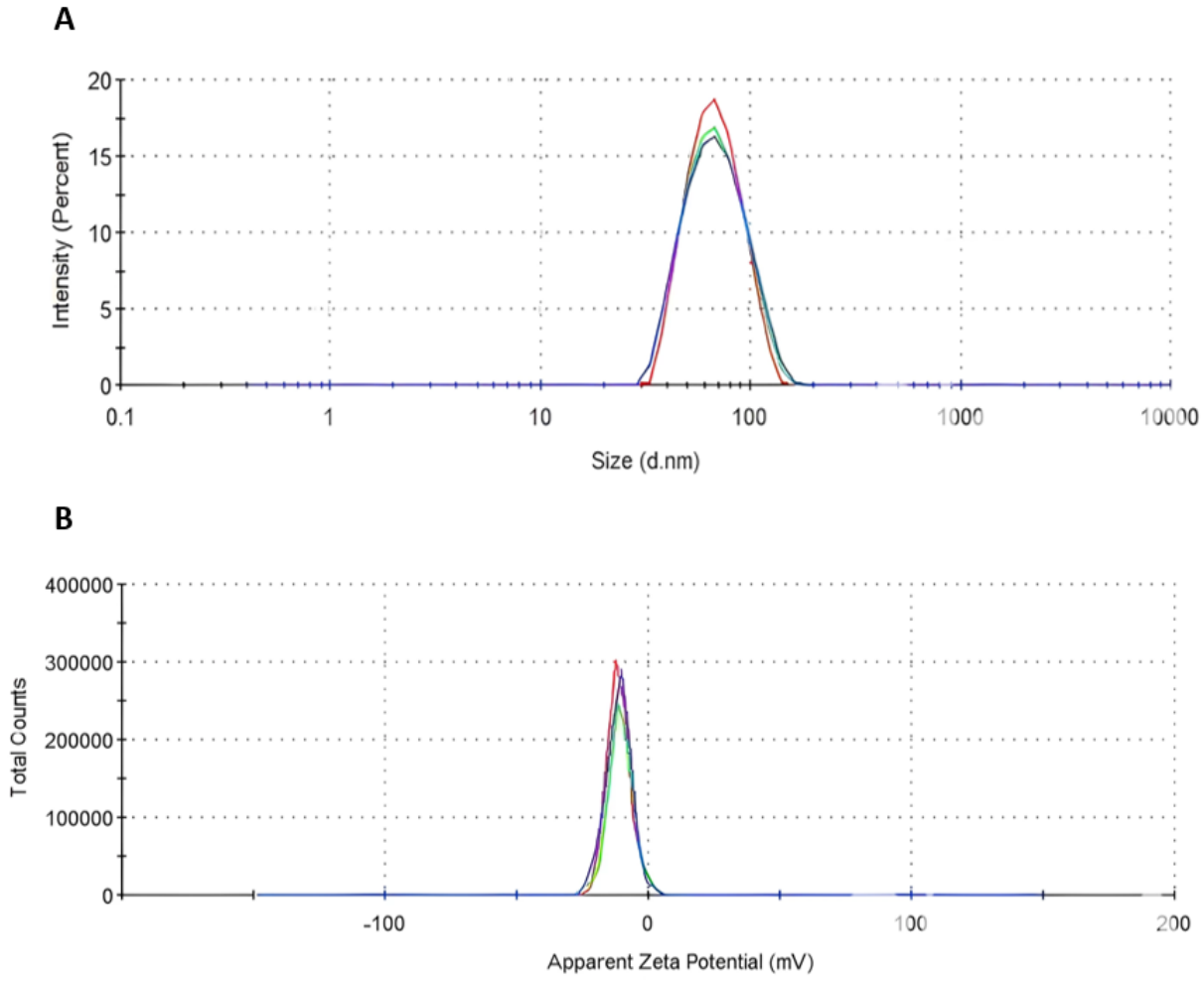
SUPPLEMENTARY MATERIALS

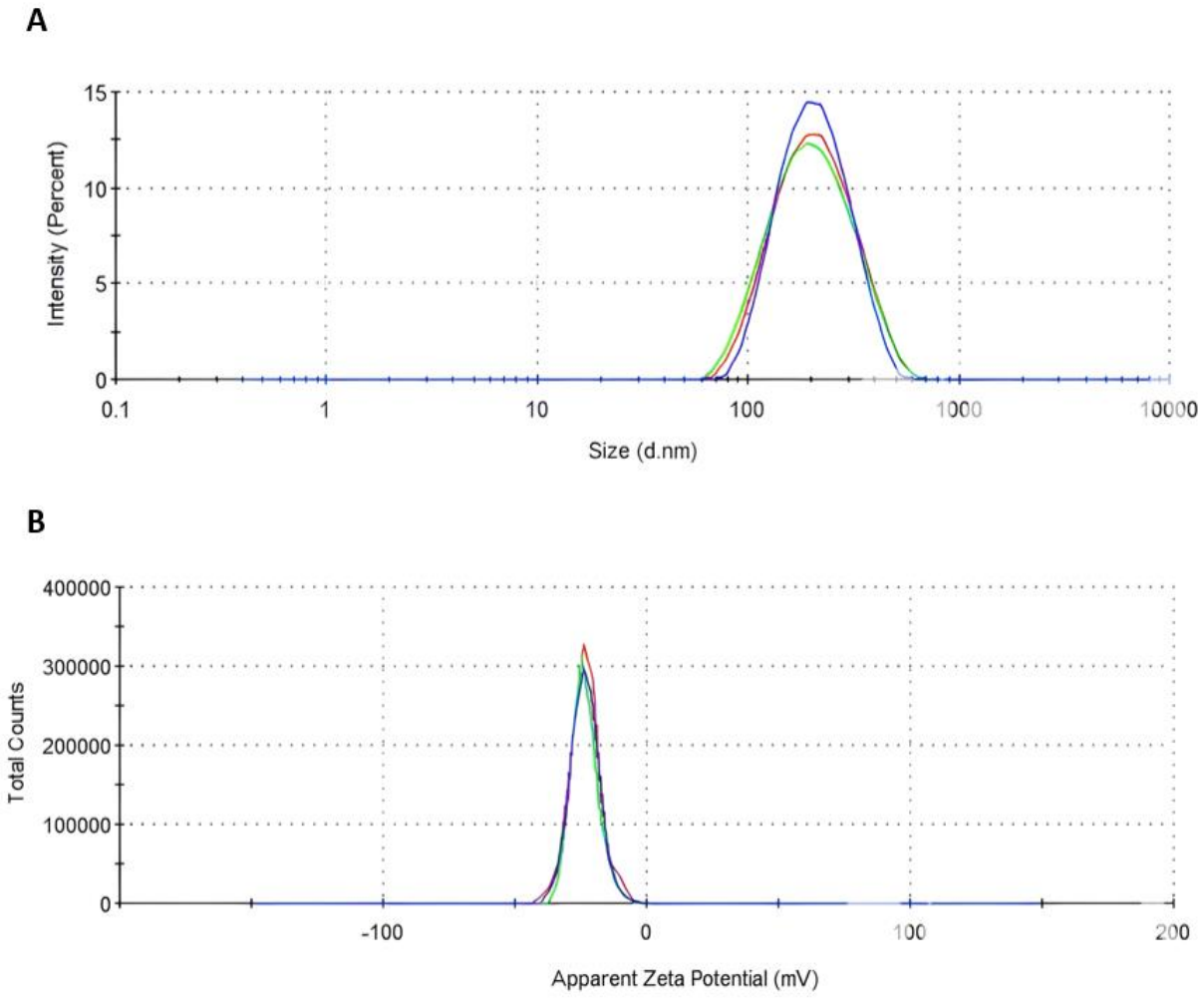
Supp. Figure 1. Size (A) and zeta potential (B) distributions of garlic-NE.



Supp. Figure 2. Size (A) and zeta potential (B) distributions of eucalyptus-NE



Supp. Figure 3. Size (A) and zeta potential (B) distributions of clove-NE



CHAPTER 8

Conclusion and future prospectives

The proposed PhD thesis broadens the knowledge about the use of botanical-based nanoformulations as insecticides in citrus pest management. Particularly, the aim of thesis was to develop and assess the biological activity of different EO-based nano-emulsions against key citrus pests including *P. citri*, *D. aberiae*, *A. gossypii* and *C. capitata*. To achieve this, first was to evaluate the best method (i.e. microfluidisation technique) that allowed us to obtain these nanoformulations with optimal composition (high EO: surfactant ratio), physical characteristics (i.e. size, PDI and surface charge) and long-term stability (Chapter 4). The study of insecticidal efficacy highlighted high mortality against all tested insects, particularly for garlic EO-based nano-formulations (Chapters 4 - 7). One critical area for future investigation is the extension of the developed methodologies to other essential oils beyond *Allium sativum*. While garlic EO has demonstrated exceptional efficacy and stability, exploring the potential of other EOs, such as clove (*Syzygium aromaticum*) or eucalyptus (*Eucalyptus camaldulensis*) could broaden the scope of these eco-friendly pest control solutions. This would involve adapting the high-energy emulsification techniques and stability protocols to accommodate the unique chemical properties of different EOs, ensuring their effective incorporation into nanoformulations. Another important direction is the evaluation of these EO-based nanoformulations under field conditions. While laboratory and controlled environment studies have provided valuable insights, field trials are essential to assess the real-world performance of these formulations. Factors such as environmental variability, UV degradation, and interaction with other agricultural inputs must be studied to optimize the formulations for practical use. However, to validate their use under real operating conditions, the impact on non-target organisms represents a key aspect that needs to be fully assessed. In this regard, the proposed thesis aimed to assess the biological activity of these botanicals-based nanoformulations towards different natural antagonists of target insects (i.e. predators and parasitoids) and honeybees (chapters 4 - 7). The results highlighted different rates of toxicity towards them depending on non-target species and the route of exposure. The developed botanical-based nano-insecticides were safe towards coccinellid predators and honeybees via residual and topical exposure (Chapters 4 - 6), meanwhile were extremely toxic towards parasitoids through the ingestion route of exposure (Chapter 7). These results suggest that further investigations on the biological activity of these substances towards non-target insects are needed, considering different factors (such as type of insect, type of formulation, application rate exposure doses etc.) in order to validate their use in IPM programs. Another important aspect that this thesis underlines is the absence or insignificant phytotoxic effects on citrus plants (Chapter 6). In addition

to the pesticidal properties, these botanical-based nano-formulations highlighted a further beneficial effect as triggered the overexpression of plant genes related to Salicylic acid (*SA*) and Jasmonic acid (*JA*) pathways involved in their natural defence mechanisms (Chapter 6). Understanding how these formulations activate systemic resistance in plants could provide a dual benefit of direct pest control and enhanced plant immunity, contributing to the adoption of more sustainable integrated pest management (IPM) strategies. Overall, botanical-based nano-insecticides represent a promising and sustainable alternative to synthetic pesticides. While this PhD thesis provides valuable insights into their potential for pest management and protection of agrobiodiversity, further research is needed to address the outstanding challenges. In addition, this PhD thesis has generated innovative and transformative knowledge by developing stable, high-efficacy essential oil (EO)-based nanoformulations, such as which not only demonstrate exceptional pest control but also minimize ecological risks, offering a sustainable and scientifically advanced alternative to conventional insecticides.

DM 1061 del 10 agosto 2021

Dottorati PON - Bando 2021 - Ciclo 37 (XXXVII)

Azione IV.4 - Dottorati e contratti di ricerca su tematiche dell'innovazione

Azione IV.5 - Dottorati su tematiche Green

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Corso di Dottorato in	**Scienze Agrarie, Alimentari e Forestali**
Ciclo	**XXXVII**
Codice borsa e n.	**DOT1647787-4**
CUP	**C35F21001320002**
Tipologia Green/Innovazione	**Green**
Titolo Progetto	**Tutela della agro-biodiversità e delle produzioni locali attraverso l'impiego di nuovi bioinsetticidi selettivi.**