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

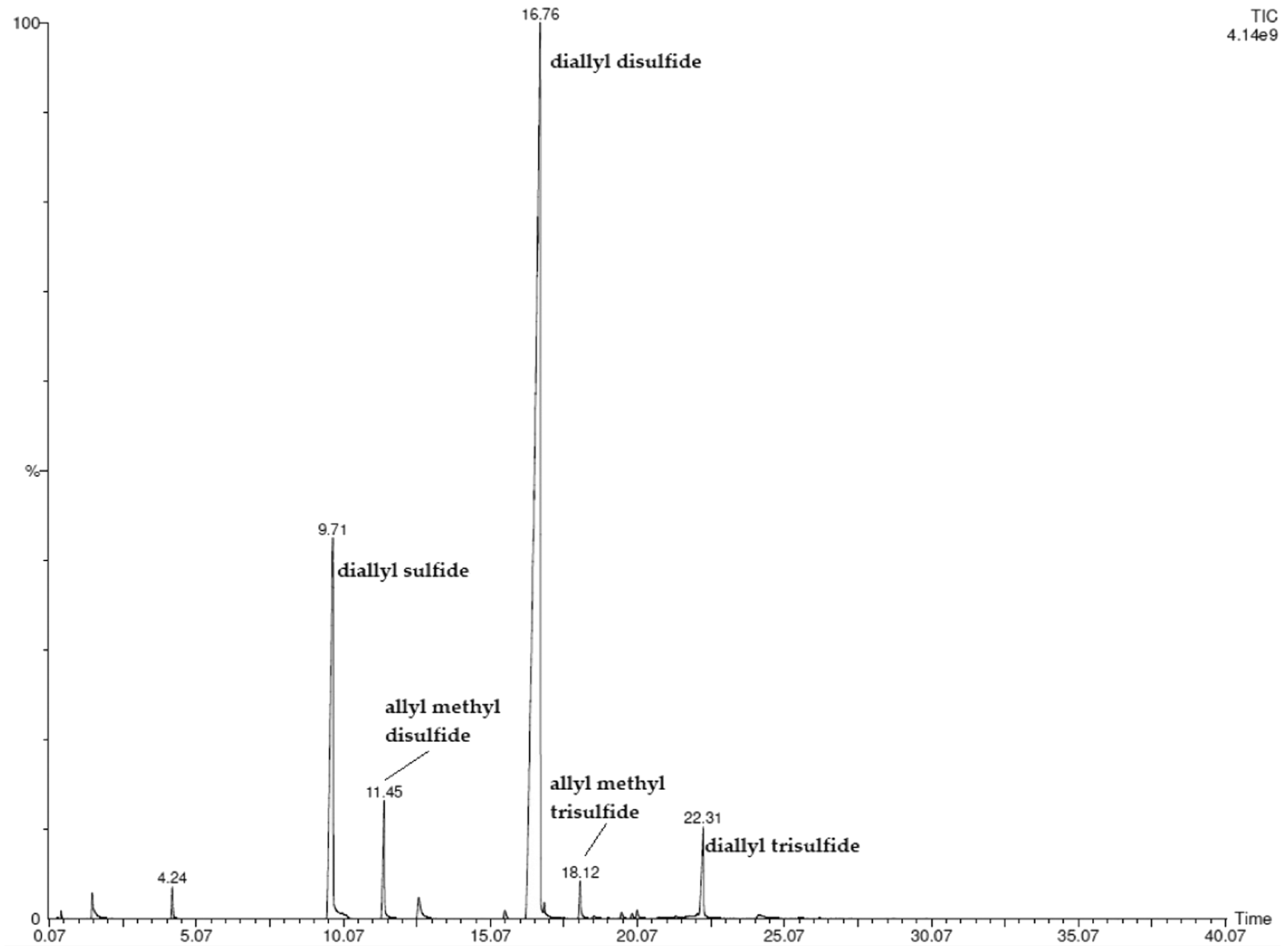

Fig. 1. GC-MS chromatogram of crude garlic EO.

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

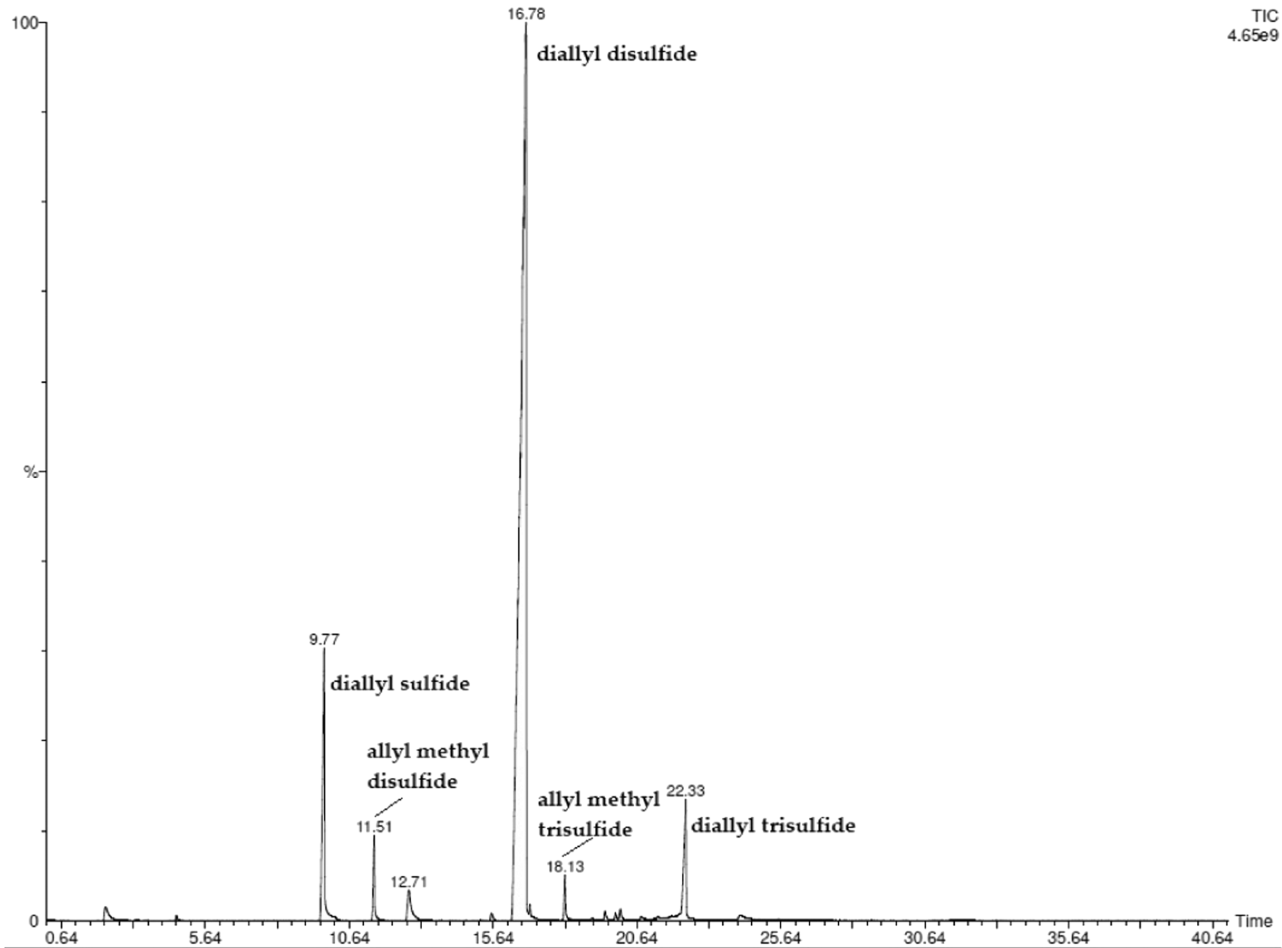

Fig. 2. GC-MS chromatogram of garlic EO in nano-emulsion.

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 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%

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.

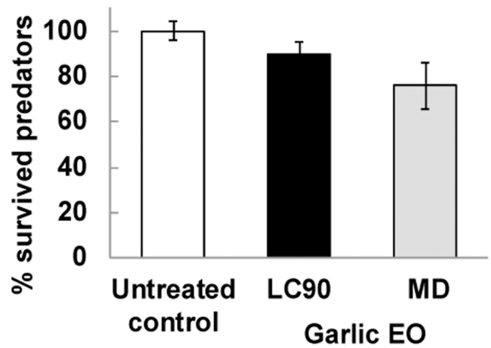

Fig. 3. Mean (±) survival values for *Cryptolaemus montrouzieri* specimens after topical contact exposure to garlic essential oil at two lethal concentrations applied for the target pest *Planococcus citri* (LC_{90} and Maximum Dose) and distilled water (untreated control). No statistical differences ($p > 0.05$) 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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