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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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 ($30 \times 30 \times 30$ 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 $25 \pm 1$ °C, $70 \pm 5\%$ 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 ($72.61 \pm 0.13$ and $73.56 \pm 0.19$ 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 ($108.3 \pm 0.35$ 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 ($0.06 \pm 0.006$—$0.187 \pm 0.01$) in SN, SH, and HPM nano-emulsions, while the RAW nano-emulsion was characterized by high PDI values ($0.912 \pm 0.07$–$0.988 \pm 0.008$). After one day, statistical differences were observed among all nano-emulsions, and the HPM method highlighted the best homogeneity ($0.06 \pm 0.006$) ($F = 129.1$; 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 *Planocccocus 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.

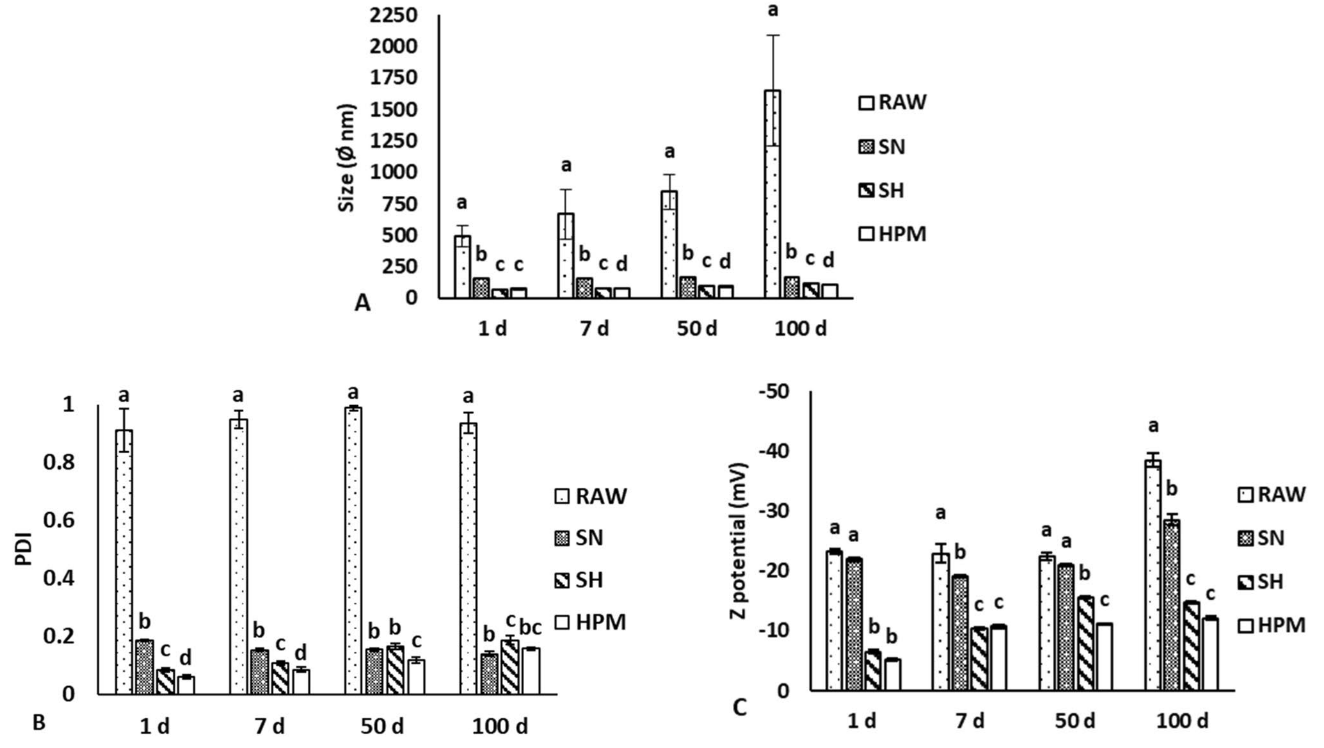

**Fig. 1** Physical properties (A = Size; B = PDI; C = ζ-potential) (mean ± SE) of nano-emulsions 1, 7, 50 and 100 days after production. Different letters indicate statistical differences among the methods within the same time (ANOVA, $P < 0.05$)

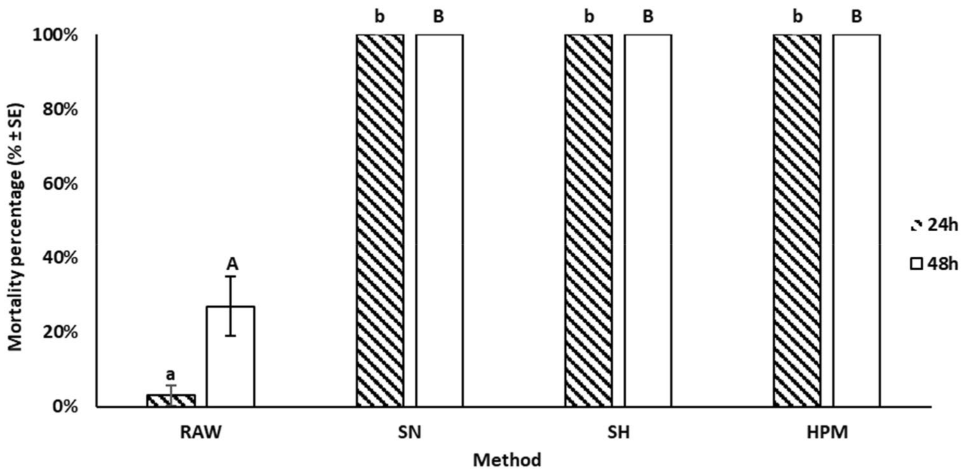

**Fig. 2** Mortality (% ± SE) 24 and 48 h after the exposure to *Allium sativum* EO-based nano-emulsions against 3$^{rd}$ instars of *Planococcus citri* in the preliminary test (2.5% of EO). Different letters indicate statistical differences among the different methods at the same exposure time (ANOVA,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

(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%

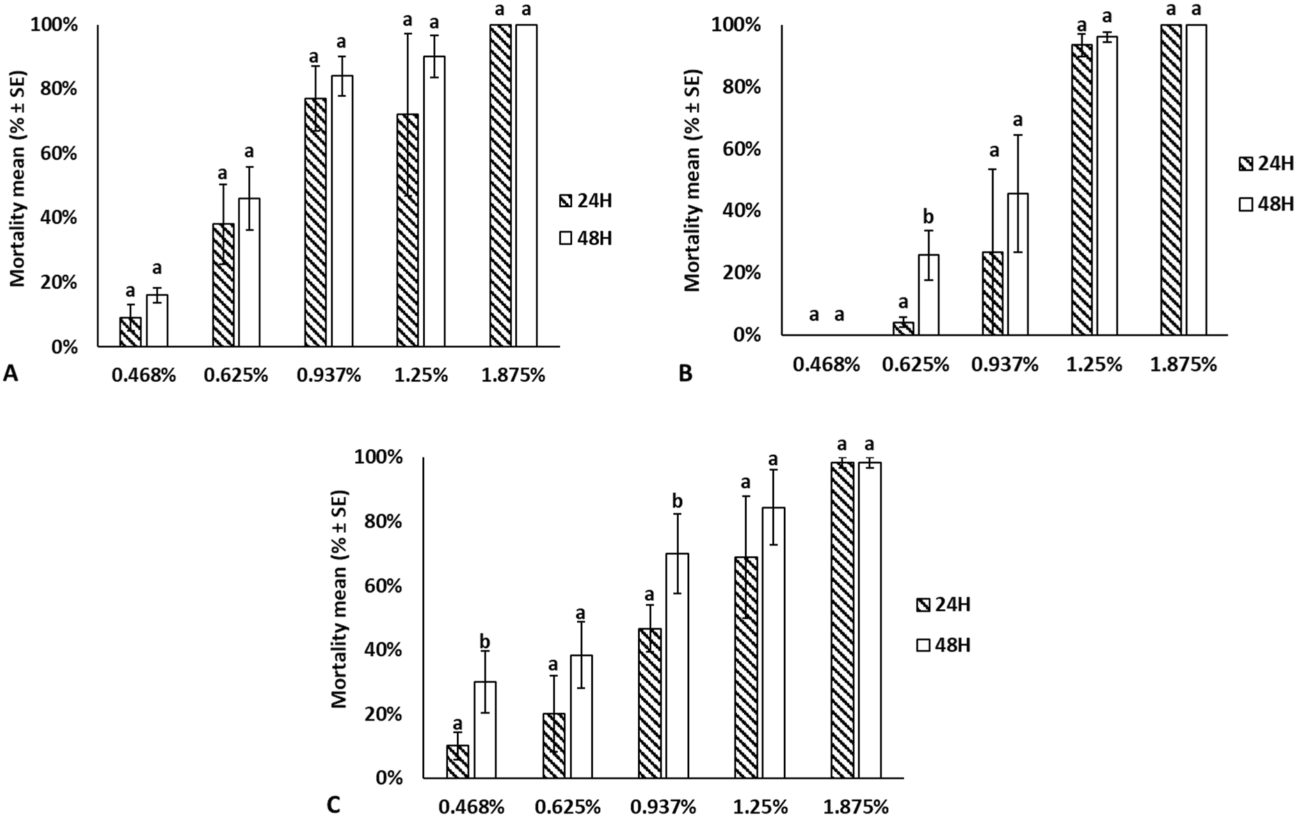

**Fig. 3** Dose–response mortality (% ± SE) caused by the developed nano-emulsions (A = SN; B = SH; C = HPM) against 3rd instars of *Planoccoccus citri* 24 and 48 h after the treatment. Different letters indicate statistical differences between different times of exposure within the same dose (EO %)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

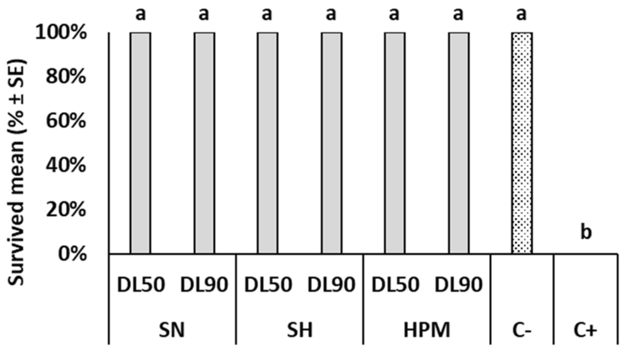

**Fig. 4** Percentage of survived *Apis mellifera* workers 96 h after exposure to nano-emulsions (SN, SH and HPM), negative control (water) and positive control (dimethoate). Different letters indicate statistical differences among the different treatments (Kruskal-Wallis test, $P < 0.05$)

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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