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Is natural always safe? Effective botanical nano-aphicide can be harmful to pollinators

Antonino Modafferi,[a] Ilaria Latella, [a] Marianna Longordo,[a] Maria Pineda, [a] Raffaele Cavaliere,[bc] Pierluigi Forlano,[b] Vincenzo Palmeri,[a] Giulia Giunti *[b] and Orlando Campolo[a]

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Among the innovative and eco-friendly solutions to conventional pesticides, nano delivery systems (*i.e.*, nanostructures and nano-emulsions) seem to be ideal candidates for botanical formulations to be used as insecticides. In this context, the proposed study aimed to formulate an *Allium sativum* EO-based nano-emulsion and to evaluate its toxicological activity against *Aphis gossypii*. Furthermore, the adverse effects of the nano-formulation on honeybees and plants were also investigated. The chemical composition of the garlic EO highlighted that the EO was composed only of sulfur compounds (95.35% of the total area). The nano-formulation (15% EO; 5% Tween® 80; 80% water w/w/v) was obtained using high-pressure microfluidization (HPM) techniques and physically characterized by dynamic light scattering (DLS). The results highlighted optimal physical properties with particle sizes ranging in the nanoscale (179 ± 1.4 nm), good polydispersity indices (PDIs), with values inferior to 0.25, and negative surface charge after 1 month of storage. The toxicological bioassays against the target pest showed high insecticidal activity with low estimated lethal doses in both residual contact toxicity (LD_{30} of 0.810, LD_{50} of 1.079, and LD_{90} of 2.171% of EO) and topical application (LD_{30} of 0.133, LD_{50} of 0.212, and LD_{90} of 0.667% of EO) after 72 h exposure. While negligible phytotoxic effects on sweet pepper plants were detected, the developed EO nano-emulsion revealed high toxicity towards honeybees through ingestion application. Overall, this study proved the high efficacy of the developed nano-biopesticide against the target pest; however, further studies are needed to fully understand the impact of these new nano-insecticides on non-target organisms in agroecosystems.

Environmental significance

Recently, nanotechnologies have found wide application for biopesticide formulations, since these allow several shortcomings of botanical extracts to be overcome while improving their effectiveness against target pests. The safety of nano-bioinsecticides toward both the environment and non-target organisms is not questioned and generally assumed. Nevertheless, nano-systems could deeply change the biological activity of botanical active substances, affecting either target or non-target species, including pollinators. Here, a nano-emulsion containing garlic essential oil showed good insecticidal activity against a target aphid species, although it also caused severe mortality toward honeybees by ingestion. In this scenario, a deeper understanding of the ecotoxicological impact of nano-biopesticides is needed to correctly design integrated pest management programs avoiding negative effects on pollinator populations.

1. Introduction

Aphis gossypii (Hemiptera: Aphididae), commonly known as the cotton aphid, is a polyphagous species that causes direct damage such as plant weakening, leaf yellowing, wilting, and the secretion of honeydew, which promotes sooty mold and hinders photosynthesis, and indirect damage (*i.e.*, pathogen transmission) to several plant families (*i.e.* Rutaceae, Malvaceae, Cucurbitaceae, *etc.*).[1–3] To date, the main control strategies are based on the use of conventional synthetic pesticides, which resulted in various consequences on the environment and human health, and also negative effects on several non-target organisms, including pollinators, natural antagonists, aquatic organisms, and invertebrates.[4,5]

Among the green solutions, botanicals such as aqueous extract, oil, and essential oils (EOs) stand out as ideal candidates to replace conventional pesticides due to their proven insecticidal activities.[6–8] In this regard, several authors have well documented the effectiveness of EOs in managing aphids.[9–13] As reported by Koorki *et al.* (2018),[14] *Eucalyptus camaldulensis* Dehn., *Rosmarinus officinalis* L., and

[a] *Department of AGRARIA, University of Reggio Calabria, Loc. Feo di Vito s.n.c., Reggio Calabria 89122, Italy*
[b] *Department of Pharmacy, University of Salerno, Via Giovanni Paolo II 132, Fisciano 84084, SA, Italy. E-mail: ggiunti@unisa.it*
[c] *Department of Agricultural, Forest, Food and Environmental Sciences, University of Basilicata, Via dell'Ateneo Lucano 10, Potenza 85100, Italy*

Ferula assa-foetida L. EOs showed repellency and fumigant toxicity against 2-day-old nymphs of *A. gossypii* 24 hours after exposure with estimated lethal concentrations $(LC)_{50}$ of 21.02, 19.28, and 4.64 μL L^{-1} air, respectively. Furthermore, several EOs could significantly alter nymphal development, as well as adult longevity, fecundity, and the amount of honeydew produced by this pest.[15] Moreover, Albouchi *et al.* (2018)[16] investigated the biological activity of an EO extracted from *Melaleuca styphelioides* Sm. against three citrus aphids, including *A. gossypii*, *Aphis spiraecola* Patch, and *Myzus persicae* (Sulzer) (Hemiptera: Aphididae), highlighting that this EO exhibited effective fumigant and contact toxicity against all test species, although *A. gossypii* was the least susceptible species.

Among botanicals, *Allium sativum* L., commonly known as garlic, has been widely used in pest control due to its high toxicity. In this context, the topical application of garlic oil against adult *A. gossypii* exhibited good insecticidal activity with an estimated LC_{50} and LC_{90} of 3302 and 10 553 ppm, respectively, 48 hours after exposure.[17] Using the same application method, Aiad *et al.* (2019)[18] highlighted high insecticidal activity against *A. gossypii* with an estimated LC_{50} of 0.59 mg L^{-1} and LC_{90} of 1.24 mg L^{-1}. Furthermore, the application of 10 μL of garlic oil led to high mortality (100%) against *M. persicae*.[19] In addition, the application of garlic EO (3% concentration) could reduce the aphid population by 100% after 1 week of treatment.[20]

Despite the high efficiency of these botanicals in pest management, some drawbacks (*i.e.*, flammability, poor solubility in water, rapid degradation of the active ingredient, and volatility) limit their use under real-operating context.[21,22] To overcome these drawbacks, in the last decade, the use of nanotechnologies to develop nano-delivery systems (*i.e.*, nanostructures and nano-emulsions) has increased the interest of researchers.[23–25] The high biological activity of innovative nano-insecticides has been well documented against several pests.[26–30] For instance, Tortorici *et al.* (2022)[31] developed three different nanostructured lipid carriers (NLCs) loaded with different EOs (*i.e.*, lavender, rosemary, and peppermint) and tested the biological activity against *A. gossypii*, *Spodoptera littoralis* (Bois) (Lepidoptera: Noctuidae), and *Phthorimaea* (Tuta) *absoluta* (Meyrick) (Lepidoptera: Gelechiidae). The results highlighted good insecticidal activity for all developed EO-NLCs against *A. gossypii* with values (% aphid survival) lower than <20%. In contrast, the formulations did not particularly affect the other target pests. Similarly, NLCs individually loaded with two EOs showed good insecticidal activity against *Culex pipiens* L. (Diptera: Culicidae) with an estimated LC_{50} of 251.71 and 278.63 ppm and LC_{90} of 637.52 and 825.55 for the fennel-NLC and green tea-NLC, respectively. Other authors highlighted the high biological activity of EOs in nano-emulsified formulations.[32–35] As described by Kavallieratos *et al.* (2022),[36] a *Carlina acaulis* EO-based nano-emulsion (1000 ppm concentrated) exhibited good insecticidal activity (mortality rate of 93.9% and 98.9%) against *Tribolium castaneum* (Herbst) and *Tribolium confusum* du Val (Coleoptera: Tenebrionidae), respectively. Similarly, Taktak *et al.* (2022)[37] developed different EO-based nano-emulsions (*i.e.*, cypress, lavender, lemon eucalyptus, and tea tree EO), which reported significant bioactivity with LC_{50} values ranging from 57.10 to 180.70 mg L^{-1} 48 hours after exposure.

In this context, this study aimed to develop a highly stable garlic EO-based nano-emulsion using the high-pressure microfluidization technique and to establish its biological activity on target and non-target organisms. The stability over time (up to 1 month) of the developed formulation was evaluated by dynamic light scattering analysis (DLS), and the particle size, the polydispersity index (PDI), and surface charge were assessed. The toxicological activity of the nano-emulsion against *A. gossypii* adults was investigated using two different methods, residual contact and topical application, and the lethal doses (LDs) were estimated. The estimated LDs against the target pest were used to evaluate adverse effects on non-target organisms, using ingestion toxicity against *Apis mellifera* L. (Hymenoptera: Apidae) and phytotoxicity trials on sweet pepper plants.

2. Materials and methods

2.1 Biological materials

The *A. gossypii* colony was reared for several generations on sweet pepper plants at the Entomology laboratories of the Department of Agriculture, University "Mediterranea" of Reggio Calabria, Reggio Calabria, Italy. The original colony was sourced from an organic citrus orchard (cv Clementine comune) located in Calabria (southern Italy). Rearing was carried out in BugDorm® cages under controlled environmental conditions (26 ± 1 °C, 65 ± 2% RH, and a 16 : 8 h light : dark photoperiod) to optimize colony development.

For toxicity trials, 1- to 2-day-old workers of *Apis mellifera ligustica* Spinola (Hymenoptera: Apidae) were collected from the experimental apiary of the same department. The bee colonies did not receive chemical treatments for at least three months prior to the experiments.

Sweet pepper plants used for both the aphids' rearing and the bioassays were cultivated from seeds in organic soil inside a climate-controlled chamber (25 ± 1 °C, 70 ± 5% RH, 14 : 10 h light : dark photoperiod). Plants that reached a height of 15–20 cm and developed 15 ± 1 leaves were used in the experiments.

2.2 Chemical characterization of garlic EO

Garlic EO was purchased from Esperis S.p.A. (Milan, Italy) and chemically analyzed using a gas chromatography-mass spectrometry (GC-MS) device, following the methods described by Giunti *et al.* (2019).[38] 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 the 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 was injected at a split ratio of 1 : 30. The identification of peaks was made using computer matching against commercial libraries (NIST 05, Wiley FFNSC, and ADAMS), comparing linear retention indices (LRIs). The LRIs were calculated using the formula of Van den Dool & Kratz (1963),[39] by comparing the retention times of the compounds to be identified with those of a standard mixture of alkanes (C8–C20 saturated alkane standard mixture, Supelco®, Bellefonte, PA, USA), which was analysed in GC-MS under identical operating conditions to the sample.[39–44]

2.3 Garlic EO-based nano-emulsion development and physical characterization

The garlic EO-based nano-emulsion was developed using a top-down approach according to the method described by Modafferi *et al.* (2024).[45] In detail, garlic 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 achieve a homogeneous oily phase. Double-distilled water was then added to the oily phase (ratio 4 : 1 v : w) and mixed for 5 min to obtain a raw emulsion (ratio 3 : 1 : 4 w : w : v for EO, Tween, and water, respectively). The obtained raw emulsion was homogenized using a high-pressure microfluidizer (HMP) device (LM20 Microfluidizer™ Processor, USA) with the pressure set at 30 000 PSI. The process was repeated five times, and to avoid EO degradation, the interaction chamber and heat exchanger were submerged in an ice bath. The obtained nano-emulsion was stored inside aluminum bottles and kept at 4 °C until the end of the experiments.

The developed garlic EO-based nano-emulsion was physically characterized using dynamic light scattering (DLS) analysis using a Zetasizer device (Zetasizer Nano, Malvern®). Specifically, the particle size (nm), polydispersity index (PDI), zeta potential (ζ-potential), and stability over time (*i.e.*, 24 hours, 1 week, and 1 month) were estimated. The analyses were conducted by diluting the developed formulation in double distilled water (ratio 1 : 200 v : v). For measurements, 1 mL and 0.75 mL of diluted nano-emulsion were used to assess the size and surface charge, respectively.

2.4 Biological activity against the target pest

The insecticidal activity of the developed garlic EO-based nano-emulsion was evaluated against *A. gossypii* adults using two different methods (*i.e.*, residual contact toxicity and topical application) in order to simulate real-world operating conditions. In both experiments, a total of six replicates of ten adult specimens were treated with different garlic EO-based nano-emulsion dilutions. Pure water and dimethoate (ROGOR® L40) water solution at label dose (0.1%) were used as negative (C−) and positive control (C+) treatments, respectively. Garlic EO-based nano-emulsion dilutions were obtained by mixing the required amount of nano-emulsion in double-distilled water (w : v). All the experiments were carried out using the same rearing conditions (26 ± 1 °C, 65 ± 2% RH, and a photoperiod of 16 : 8 h L : D) (see section 2.1). In both trials, mortality was checked 48 and 72 hours after exposure. Insects were considered dead if they did not move or were unable to walk after stimulation with a fine brush.

Residual contact toxicity bioassays were conducted following the leaf-dip method. Specifically, circular sweet pepper leaves (Ø: 3.9 cm) were individually immersed for 15 s in different nano-emulsion dilutions (*i.e.*, 0.625, 0.93, 1.25, 1.87, and 2.5% of EO), C−, and C+, dried at room temperature (25 ± 1 °C), and placed inside ventilated plastic arenas (Ø: 4 cm; v: 50 mL). Subsequently, the specimens were gently placed on the treated surface, and the arenas were kept under the above-mentioned climatic conditions.

The topical application bioassay was performed using a professional hand sprayer (1 L Volpitech, Volpi®, Italy). A total of ten garlic EO-based nano-emulsion dilutions (*i.e.*, 0.111, 0.156, 0.233, 0.313, 0.465, 0.625, 0.93, 1.25, 1.87, and 2.5% of EO), C−, and C+ were tested. Infested sweet pepper plants were individually sprayed with the different treatments until run-off and left to dry under laboratory conditions (25 ± 1 °C). The treated insects were then carefully transferred with a fine brush to untreated leaf dishes' surface (Ø: 3.9 cm) and then incubated under the above conditions.

2.5 Toxicological evaluation towards honeybees

The impact of the developed garlic EO-based nano-emulsion towards honeybees was evaluated using the oral administration method as described by Medrzycki *et al.* (2013).[46] The ingestion route was preferred over other standard application methods (*i.e.*, topical and residual contact), because it was the most adequate to test acute toxicity against honeybees when considering *A. gossypii* as target pest species. Indeed, aphids can produce honeydew, a high-value food source foraged by honeybees, which can be contaminated by insecticide applications.

A total of five treatments were performed using the estimated LD_{30}, LD_{50}, and LD_{90} (0.810, 1.079, and 2.171% of EO, respectively) obtained in residual contact toxicity bioassays (see Table 1), sucrose/water solutions (30% w/v) as negative controls (C−), and dimethoate (ROGOR® L40) at label dose (0.1%) as a positive control (C+). The desired LDs and C+ concentrations were obtained by diluting the required amount of nano-emulsion and active ingredient (a.i.), respectively, in a sucrose/water solution (30% w/v). The bees were collected and anesthetized with carbon dioxide. Then, ten specimens were gently placed inside ventilated cup-shaped hoarding arenas with a removable base, and two feeding devices containing the desired solution as described by Williams *et al.* (2013).[47] The trials were conducted under constant climatic conditions (25 ± 1 °C and 50 ± 5% RH). Each treatment was performed eight times, and the mortality

Table 1 Estimated lethal doses of the garlic EO-based nano-emulsion against *A. gossypii* 48 and 72 hours after the exposure in both methods. Values were considered significantly different if their 95% confidence limits did not overlap

LD[a]	Method	Time (h)	Estimate (EO dose%)	95% confidence limits	
				Lower bound	Upper bound
LD_{30}	Residual[b]	48	0.997	0.878	1105
		72	0.810	0.699	0.907
	Topical[c]	48	0.148	0.073	0.218
		72	0.133	0.070	0.187
LD_{50}	Residual	48	1.360	1.235	1.504
		72	1.079	0.970	1.189
	Topical	48	0.263	0.168	0.365
		72	0.212	0.140	0.283
LD_{90}	Residual	48	2.904	2.459	3.705
		72	2.171	1.892	2.631
	Topical	48	1.086	0.724	2.308
		72	0.667	0.474	1.248

[a] Lethal dose. [b] Residual contact toxicity method. [c] Topical application method.

of the specimens was recorded 4 hours after the treatment. The bees were considered dead if they were not able to fly or move after stimulation with a fine brush.

2.6 Phytotoxicity assessment on sweet pepper plants

The phytotoxic effects of the developed garlic EO-based nano-emulsion were evaluated on sweet pepper plants. In detail, plants, 15–20 cm in height, were individually treated using a hand sprayer until run-off and subsequently transferred to separate climate chambers (one per treatment). All plants were maintained under the same growth conditions (*i.e.*, 25 ± 1 °C, 70% RH, and a 14 : 10 h light : dark photoperiod). A total of four treatments were performed using the estimated LD_{30}, LD_{50}, and LD_{90} (0.810, 1.079, and 2.171% of EO, respectively) obtained in the residual contact toxicity bioassays (see Table 1) 72 hours after *A. gossypii* exposure. Each treatment was replicated six times, and the phytotoxic effects were evaluated 1, 2, 5, and 10 days after plant treatment.

Phytotoxicity was evaluated through the calculation of the phytotoxicity index (Pi), a quantitative/qualitative index which includes both the number of damaged leaves and the severity of the damage. The Pi ranges from 0 (no damage) to 1 (dead leaves), and it has been calculated as described by Campolo *et al.* (2017):[48]

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

where DL is the number of damaged leaves for each damage severity class j; TL is the total number of leaves treated; 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.

2.7 Data analysis

Data were analyzed using IBM® SPSS® Statistics 2v.23 (IBM Corp. Released 2015. Armonk, NY, USA). All data met the assumptions required for parametric testing, including normality and homoscedasticity of variance ($p > 0.05$). Differences in the physical characteristics over time were analyzed using analysis of variance (ANOVA) with the particle size and polydispersity index (PDI) as dependent variables, and the times used as fixed factors. Multiple comparisons were conducted using Tukey's HSD *post hoc* test.

Mortality data in residual contact toxicity and topical bioassays against *A. gossypii* were corrected for control mortality using Abbott's formula.[49] The results obtained 48 and 72 h after the exposure in both bioassays fitted with the Probit model, and the LDs and their 95% confidence limits were estimated.

Differences among the different treatments on non-target organisms (*i.e.*, honeybees and sweet pepper plants) were analyzed using ANOVA, and multiple comparisons were assessed using the Duncan test.

3. Results

3.1 Garlic EO chemical composition

The chemical characterization of garlic EO is shown in SI 1. Specifically, twenty-four out of forty-four compounds were detected, accounting for 95.35 percent of the total calculated area. The EO was rich in sulfur compounds, with diallyl disulfide (27.41%), diallyl trisulfide (21.45%), diallyl sulfide (16.32%), diallyl tetrasulfide (11.20%), and 8-methyl-4,5,6,9-tetrathia-1,11-dodecadiene (7.52%) being the five most abundant detected compounds, representing more than 80% of the total area calculated.

3.2 Physical characterization of the garlic EO-based nano-emulsion

The physical characteristics of the developed garlic EO-based nano-emulsion showed optimal properties in terms of size, PDI, and surface charge. The size of the nano-emulsion increased over time ($F = 7962.49$; df = 2; $p < 0.001$) (Fig. 1A). Specifically, 24 hours after development, the nano-emulsion exhibited very low particle size with a value of 86.03 ± 0.60 nm, while one week later the particle size increased to 130.3 ± 0.36 nm and after one month it was more than doubled (179 ± 1.4 nm) compared to the first measurement. Despite the increasing trend over time, the developed nano-emulsion remained in the nanometric range (<250 nm) until the end of the experiment. Regarding the PDI, the obtained garlic EO-based nano-emulsion showed a good particle size distribution with values always lower than 0.25 (Fig. 1B). Twenty-four hours after development, a very low PDI value (0.165 ± 0.001) was achieved. As observed for the particle size, the PDI increased over time too (F = 352.83; df = 2; $p < 0.001$), reaching values of 0.232 ± 0.006 and 0.227 ± 0.004 after 1 week and 1 month, respectively. Regarding the zeta potential results (Fig. 1C), the developed garlic EO-based nano-emulsion exhibited negative values ranging from −20.7 ± 0.31 to 28.1 ± 0.25. Furthermore, statistical differences were recorded between the different observation times (F = 676.02; df = 2; $p < 0.001$).

3.3 Biological activity against *A. gossypii*

The developed garlic EO-based nano-emulsion exhibited high insecticidal activity against *A. gossypii* adults in both methods. Experimental data collected at 48 and 72 h after the exposure fitted with the Probit model in residual contact (48 h: X^2 = 3.287; df = 3; p = 0.350 and 72 h: X^2 = 1.406; df = 3; p = 0.704) and topical application (48 h: X^2 = 3.073; df = 8; p = 0.930 and 72 h: X^2 = 4.018; df = 8; p = 0.856), and the LDs and their 95% confidence limits were estimated (Fig. 2). In both treatments (*i.e.*, residual contact and topical bioassays), no statistical differences were recorded between the exposure times (48 and 72 hours) within the same lethal dose, as their confidence limits overlapped. Conversely, statistical differences between the treatments were observed at both exposure times (*i.e.*, 48 and 72 hours) (Table 1). Specifically, topical application showed significantly higher toxicity than residual contact bioassays. Forty-eight hours after exposure, the estimated LDs (LD_{30}, LD_{50}, and LD_{90} of 0.148, 0.264, and 1.086% of EO, respectively) in the topical bioassay showed values lower than double those obtained in the residual contact toxicity bioassay (LD_{30} of 0.997, LD_{50} of 1.360, and LD_{90} of 2.904% of EO). A similar trend was observed 72 h after exposure, with LD_{30}, LD_{50}, and LD_{90} of 0.133, 0.212, and 0.667% of EO and 0.810, 1.079, and 2.171% of EO for the topical and residual contact toxicity bioassays, respectively. Complete LDs and their confidence limits in residual contact toxicity and topical application bioassays are shown in SI 2 and SI 3, respectively.

3.4 Biological activity towards honeybees

The biological activity of the developed garlic EO-based nano-emulsion toward honeybees is shown in Fig. 3. Honeybees were exposed to 0.810, 1.079, and 2.171% of EO, and the results highlighted the high insecticidal activity of the developed nano-emulsion, with mortality rates higher than ninety percent. No statistical differences were recorded among the tested garlic EO-based nano-emulsion doses and the C+ (dimethoate) group. Instead, the C− (sucrose water

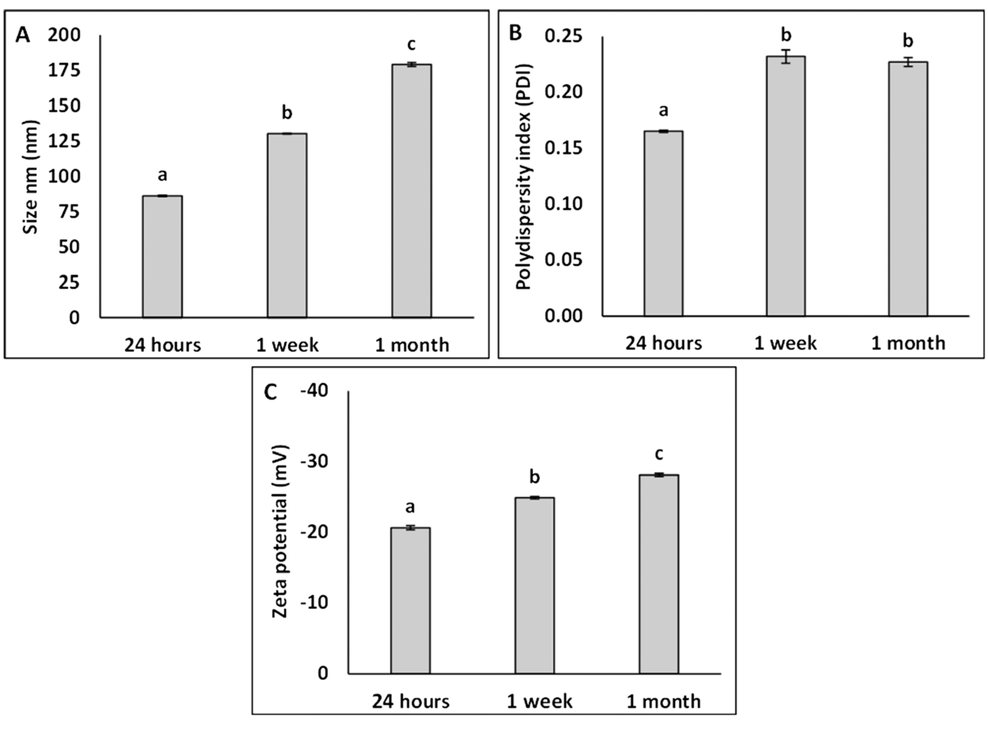

Fig. 1 Physical properties, size (A), polydispersity index (B), and zeta potential (C) of the developed garlic EO-based nano-emulsion. Values are means (±standard deviation) of three replicates. Different letters indicate statistical differences over time (ANOVA, $p < 0.05$).

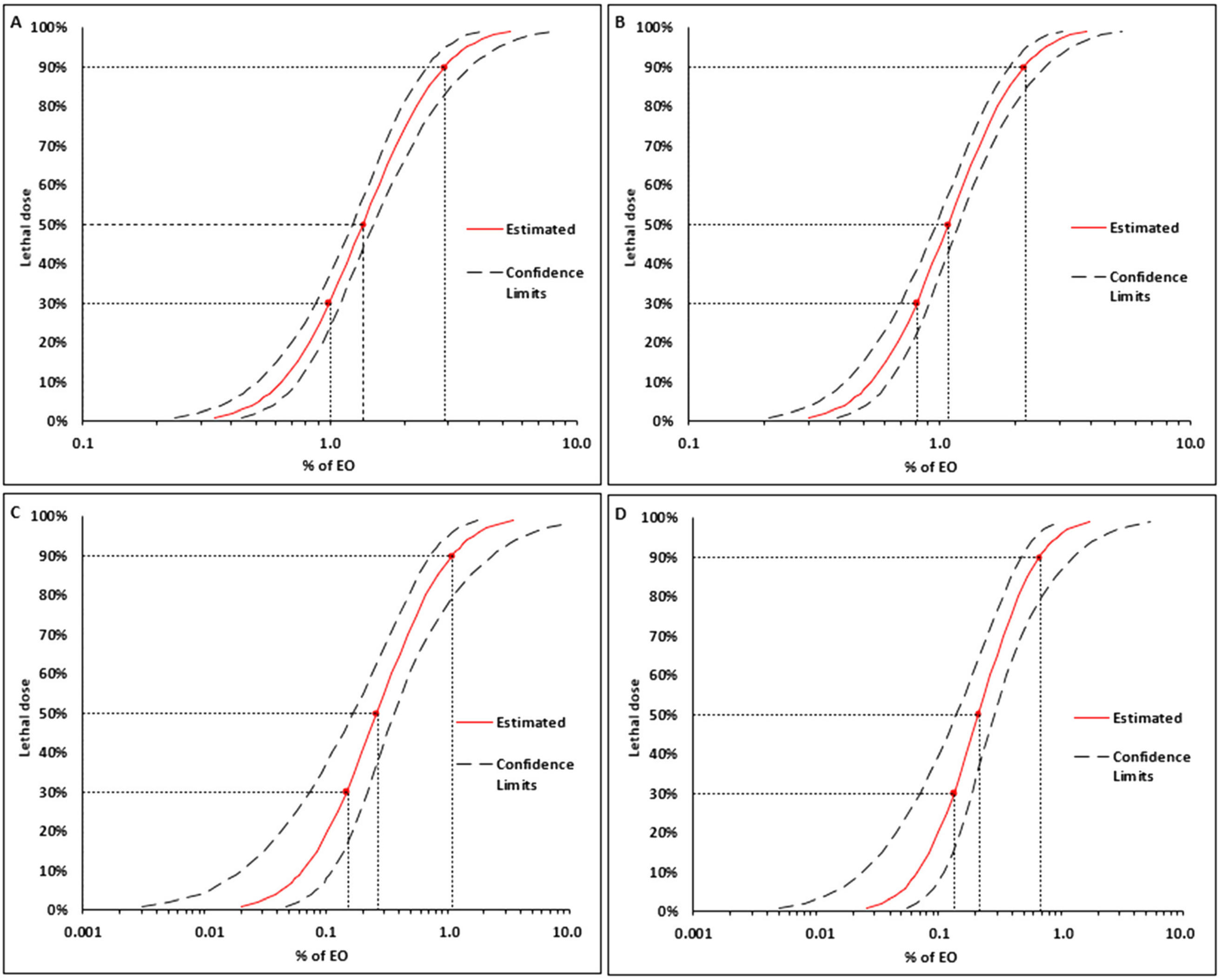

Fig. 2 Dose–response curves against the target pest: A) residual contact toxicity 48 hours after exposure; B) residual contact toxicity 72 hours after exposure; C) topical toxicity 48 hours after exposure; D) topical toxicity 72 hours after exposure. The *x*-axis is expressed in $\log_{10}$ scale.

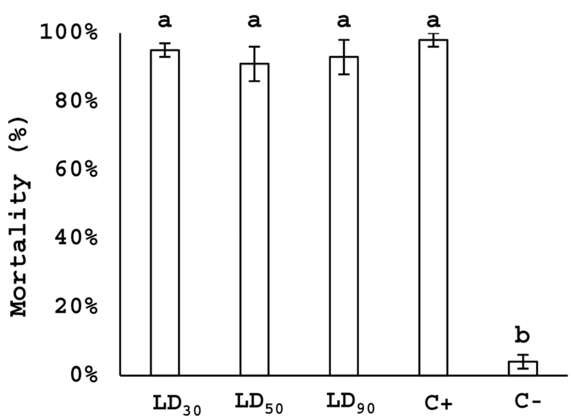

Fig. 3 Biological activity of garlic EO-based nano-emulsion doses (*i.e.*, residual contact LD_{30}, LD_{50}, and LD_{90} for aphids), positive control (C+), and negative control (C−) 4 hours after the honeybees' exposure. Values are means (±standard error) of ten replicates. Different letters indicate statistical differences among the different treatments (ANOVA, $p < 0.05$).

solution 30%) group exhibited negligible mortality with statistically different outcomes ($F = 144.37$; df = 4; $p < 0.001$) compared to other treatments (*i.e.*, LD_{30} of 0.810, LD_{50} of 1.079, LD_{90} of 2.171% of EO, and C+).

3.5 Phytotoxicity to plants

The results of the phytotoxic bioassays on sweet pepper plants are shown in Fig. 4. Plant damage was estimated through the calculation of the phytotoxicity index (Pi), by accounting for both the severity and the ratio of injured leaves due to the treatment. In general, the Pi showed an increasing trend for all tested doses (*i.e.*, LD_{30}, LD_{50}, and LD_{90} for residual contact against aphids) up to 5 days after treatment, which decreased 10 days after the treatment with Pi values less than 0.05 for all the treatments. Conversely, no phytotoxicity was observed in the control groups (Pi = 0) until the end of the experiment. In detail, 1 day after the treatment, only LD_{90} showed a Pi value (0.072 ± 0.027)

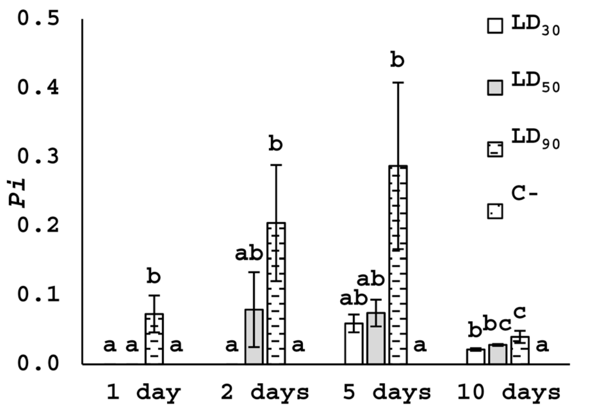

Fig. 4 Phytotoxicity index (Pi) on sweet pepper plants after different times of exposure (1, 2, 5, and 10 days) with different garlic EO-based nano-emulsion lethal doses (LD_{30}, LD_{50}, and LD_{90}) and the negative control (C−). Values are means (±standard error) of six replicates. Different letters indicate statistical differences among the different treatments at the same time (ANOVA, $p < 0.05$).

statistically different (F = 6.644; df = 3; p = 0.003) compared to those of LD_{30}, LD_{50}, and C− which did not show phytotoxic effects (Pi = 0). Similarly, 2 days after the treatment, the LD_{90} showed the highest Pi value (0.205 ± 0.084), which was significantly higher (F = 3.911; df = 3; p = 0.025) compared to LD_{30} and C− treatment (Pi = 0), while the LD_{50} group did not show statistical differences with other treatments (Pi = 0.079 ± 0.054). After 5 days, LD_{90} treatment provoked a Pi value (0.287 ± 0.012) more than double compared to Pi values of LD_{30} and LD_{50} groups (0.059 ± 0.013 and 0.074 ± 0.019 respectively); however, statistical differences were recorded only between LD_{90} and C− (Pi values of 0.287 ± 0.012 and 0, respectively) (F = 3.207; df = 3; p = 0.045). At the end of the experiments (10 days), very low Pi values (Pi < 0.05) were recorded in all the treatments, and statistical differences (F = 12.77; df = 3; $p < 0.001$) were noted among C−, LD_{30}, and LD_{90} groups (0, 0.021 ± 0.002, and 0.039 ± 0.009 Pi, respectively).

4. Discussion

The analysis of the chemical composition revealed that garlic EO was rich in sulfur compounds (95.35%), with diallyl disulfide (27.41%) and diallyl trisulfide (21.45%) being the most abundant compounds detected. These findings are consistent with our previous study, which also identified these two compounds as the major constituents of garlic EO.[50,51] Moreover, other studies have reported that different varieties of garlic were similarly rich in these organosulfur compounds.[52,53] However, it should be noted that the composition of EOs can be influenced by several factors, including the drying method, extraction process, and analytical techniques used.[54,55]

In this study, a highly stable garlic EO-based nano-emulsion with a high amount of a.i. (15% of EO) was obtained using an HPM process. Generally, nano-emulsions can be obtained using different approaches, like the one used in this study (HPM) or sonication and high-pressure homogenization (HPH).[56,57] Among these, HPM is the most efficient due to the high impact forces and the ability to use it in large-scale production.[58,59] Furthermore, the HPM process was well known to produce EO-based nano-emulsions with particle sizes ranging in the nanoscale (<250 nm) and low PDI values (<0.3).[60,61] In this context, the proposed garlic EO-based nano-emulsion showed optimal physical characteristics comparable with those obtained by other authors who used the same process. As described by Xing *et al.* (2024),[62] the HPM process was able to produce different cinnamon EO-based nano-emulsions with particle size values (ranging from 86.84 ± 0.53 nm to 107.50 ± 1.56 nm) comparable to those obtained from our nano-emulsion 24 hours after preparation (86.03 ± 0.60 nm). Similarly, Dimak *et al.* (2024),[63] developed a peppermint EO-based nano-emulsion with a very fine droplet size (69.8 nm) and PDI values slightly higher than those reported for our nano-emulsions 1 month after formulation (0.277 and 0.227, respectively). Other authors, using the HPM method, developed different EO-based nano-emulsions (*i.e.*, thyme, lemongrass, cinnamon, peppermint, and clove EOs), all ranging in the nanoscale for at least 1 month, with particle size values (<160 nm for all nano-emulsions) lower than those recorded for our 1-month-old nano-emulsions (179 ± 1.4 nm).[64] However, the amount of EO used in these nano-emulsions was very low compared to the one used in this study (2.5 *vs.* 15%, respectively). The amount of EO loaded in the nano-formulations for pesticide applications should be maximized, as this is a key aspect of their effectiveness and commercial viability. Indeed, a low concentration of the a.i. may be ineffective and require large volumes of product to cover the wide areas of crop fields, which leads to storage, transport, and application challenges.[29] Another important feature of the garlic EO-based nano-emulsion developed in this study is the relatively low amount of co-formulant (EO : surfactant ratio 3 : 1) employed to achieve stabilization. It is well known that high amounts of co-formulants can promote the development of EO-based nano-emulsions with very small droplet sizes and PDI values close to zero. Nevertheless, excessive use of these substances negatively affects the plants, leading to plant growth and cell membrane permeability alterations, increased contaminant uptake, and damage to vegetable tissues. For this reason, minimizing the amounts of co-formulants used is essential to preserve the compatibility of the formulation with crop plants.[65–67] The ζ-potential is one of the most important parameters for assessing the colloidal stability of nano-emulsions. Generally, ζ-potential values around ±30 mV are considered optimal for ensuring system stability.[68,69] Our results fall within this range with ζ-potential values from 20.7 ± 0.31 to 28.1 ± 0.25, confirming high colloidal stability. Moreover, the use of non-ionic surfactants (*i.e.*, Tween 80) significantly contributes to stability through a combination of electrostatic and steric repulsion. Under these conditions, nano-emulsions may remain stable due to ζ-potential values of approximately ±20 mV, maintaining droplet sizes in the nanoscale range.[50,70,71]

In this study, the developed garlic EO-based nano-emulsion showed high insecticidal activity against *A. gossypii* adults with low estimated LD values (LD_{50} and LD_{90} of 1.079 and 2.171% of EO, respectively) in residual contact application and LD values more than halved in topical application (LD_{50} of 0.212% of EO and LD_{90} of 0.667% of EO) 72 h after exposure. To the best of our knowledge, this is the first study that proves the high insecticidal activity of garlic EO formulated in nano-emulsion against the cotton aphid. However, the biological activity of other EO-based nano-emulsions against this pest was investigated in previous studies.[72,73] As an example, Laudani *et al.* (2022)[74] developed a *Citrus sinensis* EO-based nano-emulsion and evaluated its biological activity against this pest through a topical application method. Although this nano-emulsion showed good insecticidal activity after 48 h, the estimated LD_{50} and LD_{90} (1.48 and 2.86% of EO, respectively) were greater compared to the values obtained in the present study after the same exposure time (LD_{50} of 0.263% of EO and LD_{90} of 1.086% of EO). This suggests the higher insecticidal efficacy of the garlic EO-based nano-emulsion compared to the one formulated with a different a.i., *C. sinensis* EO. Similarly, two different natural pyrethrin-based nano-emulsions were able to reduce the insect population by approximately 90% up to 72 hours after exposure when topically applied.[75] The efficacy of EO-based nano-emulsions was well-proven against other aphid species. A spearmint EO-based nano-emulsion exhibited good insecticidal activity with an estimated LC_{50} of 2.87–2.81 mg mL^{-1} and an acetylcholinesterase inhibitory activity $(IC)_{50}$ of 1.66–5.34 mg mL^{-1} against *Rhopalosiphum maidis* (Fitch) and *Sitobion avenae* F. (Hemiptera: Aphididae), respectively.[76] Furthermore, other authors reported the efficacy of several EO-based nano-emulsions against *Aphis craccivora* Koch and *Aphis fabae* Scop. (Hemiptera: Aphididae).[77–80]

Despite growing interest in botanical nano-insecticides, their ecological impacts are poorly investigated. Most studies focus on metallic or synthetic nano-formulations,[81,82] highlighting the need for research on these formulations. However, many concerns for synthetic nanomaterials may not apply to botanical nano-insecticides, whose natural origin generally promotes rapid biodegradation, which in turn contributes to their selectivity toward non-target organisms (*e.g.*, pollinators, natural antagonists, and aquatic invertebrates) and facilitates the translation of promising laboratory results into practical field applications.[29,83] Nevertheless, only a few studies investigated the biological activity of these formulations on non-target organisms, and their fate in the environment is not easy to foresee. As shown by Mazzara *et al.* (2023),[84] the estimated LC_{90} (207.2 ppm) of a *Cannabis sativa* L. EO-based nano-emulsion against *Culex quinquefasciatus* Say (Diptera: Culicidae) did not significantly affect the survival (mortality <16% 48 h after the exposure) of an aquatic microcrustacean (*i.e.*, *Daphnia magna*). Similarly, a *Schinus terebinthifolius* Raddi EO-based nano-emulsion exhibited high insecticidal activity against *C. pipiens* with an estimated LC_{90} of 13.2 and 11.3 μl L^{-1} on larvae and adults, respectively. However, toxicological trials toward non-target larvivorous fish such as *Gambusia affinis* proved no significant effect on the survival (LC_{50} and LC_{90} of 3042.7 and 5614.7 μl ml^{-1}), longevity, and swimming activity of the non-target species; in addition, up to a concentration of 200 mg kg^{-1}, no mortality was observed also on *Eisenia fetida*.[85]

Concerning pollinators, a *Persea venosa* Nees & Mart EO nano-emulsion did not cause any mortality toward bees.[86] Nevertheless, the effects of these environmentally friendly formulations depend on several factors, including the type of EO and non-target insect, the application rate, the dose, and the route of exposure.[27,87] In this regard, previous research demonstrated that the estimated LD_{50} and LD_{90} for the pest *Planococcus citri* Risso (Hemiptera: Pseudococcidae) of different garlic EO-based nano-emulsions had no impact on the survival of honeybees through topical application (100% survival), while the positive control (dimethoate at 0.1%) led to complete mortality.[50] Nevertheless, pollinators and parasitoids may come into contact with insecticide residues also when foraging for food sources on flowers, as well as on honeydew produced by aphid species. In this regard, in this study the acute toxicity of the garlic nano-emulsion was tested in oral administration trials, to account for this kind of exposure. In contrast to topical toxicity trials,[50] here the toxicological results on honeybees highlighted that the garlic EO-based nano-emulsion was highly toxic when administered *via* ingestion, resulting in 100% mortality. A comparable outcome was reported by Modafferi *et al.* (2025),[88] who estimated the LDs (LD_{30}, LD_{50}, and LD_{90} of 0.69, 0.96, and 2.18% of EO, respectively) against the target pest and investigated the toxicological impact against a non-target parasitoid, *Aganispis daci* (Weld) (Hymenoptera: Figitidae), emphasizing the high insecticidal activity (100% mortality) of ingestion administration at all the tested doses. These findings indicate that the selectivity of botanical nano-formulations, particularly those based on garlic EO, requires further investigation to fully assess their bioactivity against non-target insects, including honeybees. When comparing the results from topical and oral trials, it is clear that ingestion poses a significantly higher risk to honeybee survival, whereas contact exposure appears to be safe. This suggests that the application of the garlic EO-based nano-emulsion should be avoided during crop flowering, as food resources for natural antagonists and pollinators (*i.e.*, pollen and nectar) are particularly abundant at this stage, potentially leading to greater ingestion of these harmful substances. Overall, the risks associated with insecticide use during this critical crop phase are quite well acknowledged, and its use at flowering is inhibited.[89–91] Furthermore, the presence of massive alternative food sources, as honeydew produced by aphid species, may also trigger serious damage to honeybees, as well as to several other non-target species foraging on it.

Lastly, concerning the outcomes observed in sweet pepper plants after exposure to the developed garlic EO-based nano-emulsion, low phytotoxic effects were detected, and only the estimated LD_{90} (2.171% of EO) resulted in a Pi value of about 0.3 up to 5 days, which decreased to no significant effect 10 days after treatment. Differently, a comparable nano-emulsion containing 3% of the a.i. resulted in very low Pi values (0.13 ± 0.1), although the treated plants showed less fruits per plant (<3) compared to the untreated control group (4.2 ± 0.4).[92] However, as previously outlined, the adverse effects depend on several factors, including the plant species. For instance, Ricupero *et al.* (2022)[93] demonstrated that a garlic nano-emulsion did not exhibit phytotoxic effects on tomato plants. A similar outcome was observed in citrus plants treated with a comparable nano-emulsion. In addition, garlic treatment enhanced the natural plant defense system through the expression of different genes involved in salicylic and jasmonic pathways.[51]

5. Conclusion

This study highlighted the potential effectiveness of a novel green nano-pesticide as an alternative to conventional formulations for *A. gossypii* control. The proposed garlic EO-based nano-emulsion obtained through microfluidization showed optimal physical properties with nanoscale particle size (<200 nm), low PDI values (<0.25), and negative surface charge up to 1 month after development. The result obtained in toxicological bioassays against adults *A. gossypii* showed a high mortality rate in both methods (*i.e.*, residual contact and topical application), highlighting the good efficacy of the developed nano-emulsion in the target pest control. Furthermore, minimal phytotoxic effects on pepper plants (Pi < 0.1) until the end of the experiment were highlighted. On the other hand, the garlic nano-emulsion exhibited a high mortality rate (100%) towards honeybees by ingestion. These results underline that, although this nano-emulsion is highly effective against the target pest, the adverse effects on non-target organisms need to be carefully considered to minimize their impact under open field conditions. Furthermore, our results shed light on the need for further investigations on the bioactivity of green nano-insecticides on key non-target organisms, including pollinators, before their application in agroecosystems.

Author contributions

AM, GG, and OC: conceptualization; AM, GG, and OC: formal analysis; VP: funding acquisition; AM, ML, MP, and RC: investigation; IL and PF: validation. AM: writing the original draft. All authors reviewed, edited and approved the manuscript.

Conflicts of interest

There are no conflicts to declare.

Data availability

Data for this article, including physical characterization of the nano-emulsion and toxicological trials, are available at Mendeley Data at **https://doi.org/10.17632/tbb6ggcmgs.1**.

Chemical characterization of garlic essential oil by GC-MS has been included as part of the supplementary information (SI). Supplementary information is available. See DOI: **https://doi.org/10.1039/d5en00498e**.

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