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Nanostructured lipid carriers of essential oils as potential tools for the sustainable control of insect pests

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ABSTRACT

Plant essential oils (EOs) represent a promising alternative and sustainable tool to reduce the negative environmental impact of conventional management systems, e.g., synthetic insecticides. Their practical implementation is hindered by various drawbacks, such as phytotoxicity, stability and degradation patterns. Innovative and advanced nanotechnologies, such as Nanostructured Lipid Carriers (NLCs), could help overcoming such problems. NLCs, composed by 10% w/v of lipid and 10% w/v of oil (Lavender, Rosemary, Peppermint) were prepared through PIT method. The obtained formulations showed the presence of small (200 nm) and homogeneous particles (Polydispersity Index (PDI) < 0.3). All NLCs were purified obtaining a 20% w/v EO concentration using ultracentrifugation, without any significant variation of mean size and homogeneity. The efficacy of these formulations was tested for three key insect pests with different feeding strategies: a sap-sucking (*Aphis gossypii*), a chewer (*Spodoptera littoralis*) and a leafminer (*Tuta absoluta*). NLCs loaded with EOs were diluted with distilled water at 30:70 v/v and were tested by ingestion and topical contact exposure routes. Long-term stability results obtained by Turbiscan® technology showed the occurrence of not significant (ΔBS < 20%) sedimentation phenomenon after 30 days of storage at 25 °C. Among the three EO-NLCs, Rosemary demonstrated to be the most long-term stable, as confirmed by the destabilization kinetic. The results of the bioassays showed that all the tested EO-NLCs, as well as the nanocarrier alone, caused high mortality on *A. gossypii* and significantly reduced its progeny by topical contact exposure. NLCs of Lavender and Rosemary EOs decreased the feeding activity but not the survival of *S. littoralis*. Conversely, the three EO-NLCs did not decrease the survival and the feeding activity of *T. absoluta*. Our findings suggest that NLCs can successfully deliver EOs by keeping unaltered their properties, therefore this nanotechnology could contribute to the sustainable control of arthropod pests.

1. Introduction

Plant essential oils (EOs) are highly volatile substances with complex chemical structure mostly constituted by aromatic compounds that smell intensely (Regnault-Roger et al., 2012). They are synthesized in different plant organs and structures (e.g., flowers, leaves, trichomes) and stored in secretory cells and epidermal glands (Bakkali et al., 2008). EOs play many different roles in plant-arthropod interactions (e.g., attract pollinators, mediate trophic activity of insect pests and natural enemies) and show antimicrobial and insecticide activities (Soares et al., 2019; Al-Ansari et al., 2021; Benelli et al., 2021; Du et al., 2021; Karkanis and Athanassiou, 2021; López et al., 2021). As a consequence, EOs have a key role in plant protection and can be used as alternative tools to reduce the negative environmental impact of conventional management systems (Giunti et al., 2019; Pavela et al., 2020; Andrade et al., 2021; Sciortino et al., 2021).

Unfortunately, EOs have intrinsic physicochemical characteristics that are difficult to manage and do not allow their practical use (Tian et al., 2021). For instance, EOs can cause toxicity on plants when formulated at high concentrations, but their efficacy is reduced in the field due to high degradation patterns. Moreover, EO homogeneous solutions in water are difficult to obtain and their stability is often

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complicated (Campolo et al., 2017). Nevertheless, these drawbacks could be easily overcome by nanotechnologies that are currently used in agriculture as bio-active formulations (Athanassiou et al., 2018; Pascoli et al., 2020; Falleh et al., 2021; Pavela et al., 2021).

Nanostructured Lipid Carriers (NLCs) are a second-generation nanocarriers constituted by a blend of a solid lipid with a liquid lipid that cover the deficiencies of solid lipid nanoparticles (SLNs) which are solely made by solid lipids. This new technology ameliorates the physical and colloidal stability of formulation and its resistance to ultraviolet light (UV) and oxidation (Nguyen et al., 2012). NLCs could be thus a promising tool to encapsulate EOs because this delivery system can control their release after the application, protect them from degradation and lower their toxicity on plants (Katopodi and Anastasia, 2021). Many studies have shown interesting results for EOs encapsulation in NLCs and their properties are well known and routinely used in pharmaceutics, food industry and cosmesis (Maroofpour et al., 2019; Dobreva et al., 2020; Waghule et al., 2020). In agricultural crop protection, NLCs could help in reducing insecticide doses and avoid their repeated applications. However, only few studies have reported the use of NLCs as pesticide formulations and new advances in this field are thus required (Frederiksen et al., 2003; Nguyen et al., 2012, 2016).

In this framework, the aim of the study was the development of potential bio-insecticide products made by long-term stable NLCs loaded with commercially available EOs of Rosemary, Lavender and Peppermint as intrinsic oily components (Carbone et al., 2018; Bonaccorso et al., 2021a,b). NLCs were prepared using Softisan 100 as solid lipid and a surfactant mixture composed of Kolliphor RH40 and Labrafil, selected for their biological information reported in the safety data sheet highlighting they have no negative environmental impact. We tested under laboratory conditions the efficacy of EO-NLCs in term of lethal and sublethal effects against three agricultural key pests with three different feeding strategies. The sap-sucking, *Aphis gossypii* Glover (Hemiptera: Aphididae), the chewer, *Spodoptera littoralis* (Boisduval) (Lepidoptera: Noctuidae) and the leafminer, *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae) were chosen because of their economic relevance in agricultural context (Campolo et al., 2014; Wang et al., 2017; Biondi et al., 2018; Hullé et al., 2020; Miranda-Fuentes et al., 2021; Desneux et al., 2022). The results of this study will contribute providing the potential basis for the sustainable management of agricultural pests in cropping systems.

2. Materials and methods

2.1. EO-NLCs

2.1.1. Materials

Hydrogenated Coco-Glycerides (Softisan 100) was purchased from IOI Oleo GmbH (Oleochemicals, IOI group). Oleoyl Macrogol-6 Glycerides (Labrafil) was a gift from Gattefossé Italia s.r.l. (Milano, Italy). Kolliphor RH40 was bought from BASF Italia S.p.a. (Cesano Modena, Italy). Rosemary (*Rosmarinus officinalis*) (oxygenated monoterpenes 68%; monoterpenes 28%), Lavender (*Lavandula angustifolia*) (oxygenated monoterpenes 90%; monoterpenes 3%) and Peppermint (*Mentha x piperita*) (oxygenated monoterpenes 80%; monoterpenes 9%) EOs were purchased from Esperis s.p.a. (Milan, Italy). EOs used for the NLC formulations belonged to the same extraction batch than those characterized chemically by Campolo et al. (2020a). Triglyceride caprylic-capric (Tegosoft CT, Miglyol 812) was supplied by Farmalabor (Canosa di Puglia, Italy).

2.1.2. Nanoparticles preparation

NLCs, composed as described in Table 1, were prepared through the phase inversion temperature (PIT) method (Carbone et al., 2014). Both lipid phase – composed of surfactants Kolliphor RH40 and Labrafil, and the solid lipid Softisan – and water were separately heated to 70 °C; then the liquid lipid was added to the lipid phase and the system was rapidly cooled to room temperature, obtaining nanoformulations containing Lavender (L-NLC), Peppermint (P-NLC) and Rosemary (R-NLC) EOs. Moreover, a control formulation without EO was prepared using Tegosoft (CT-NLC), generally recognized as safe (GRAS) compound and commonly used in the preparation of drug delivery systems. All the samples were purified removing the excess of surfactants through ultracentrifugation (SL16R Centrifuge, Thermo Scientific, Rodano, Italy) at 1 °C and 13,000 rpm for 2 h. The obtained pellets were vortexed (Heidolph Reax 2000, VWR, Milan, Italy) for 60 s in order to resuspend the nanoparticles.

Table 1
Quali-quantitative composition (% w/v) of the prepared NLCs.

	% w/v
Kolliphor RH40	6.0
Labrafil	7.5
Softisan 100	10.0
Liquid oil[a]	10.0
Water	66.5

[a] essential oil (Lavender, or Peppermint or Rosemery) or commercial Tegosoft CT.

2.1.3. Photon correlation spectroscopy (PCS)

Photon Correlation Spectroscopy (PCS) was used to determine mean particle diameter (Zave), polydispersity index (PDI) and ZP of the diluted samples (1:10 in deionized water) measured twenty-four h after their preparation. In order to perform this analysis, a Zetasizer Nano ZS90 (Malvern Instruments, Malvern, UK) was used, with a detection angle of 90°, a temperature of 25 °C and a 4 mW He–Ne laser operating at 633 nm. The obtained values were reported as the mean of at least three measurements ± SD.

2.1.4. Entrapment efficiency

The amount of each EO encapsulated into the NLC system was quantified as entrapment efficiency (EE%) using the following equation:

EE% = ((total amount of oil used-amount of unencapsulated oil)) / (total amount of oil used) × 100 (1).

Each NLC was centrifuged at 13,000 rpm, 1 °C for 2 h. The supernatants were separated and diluted with an ethanol–water mixture (75:25) before the spectrophotometric analysis performed using an UV–vis spectrophotometer (UH5300 UV-Visible Double-Beam Spectrophotometer, Hitachi Europe, Milan, Italy). Different wavelengths were used: Rosemary EO was analysed at 228 nm, Lavender and Pepprmint at 230 nm.

2.1.5. Stability studies by turbiscan® AG station

Turbiscan® AGS (Formulation, L'Union, France) is an apparatus composed of an Aging Station, with a robot and three thermoregulated blocks for the storage of the samples, and a Turbiscan Lab Expert, which uses a multiple light scattering to analyse light dispersion through a concentrated suspension. The infrared light source has a wavelength of 850 nm; the synchronous transmission (Tr) detector receives the light ray that crosses the sample at 180°, while the back scattering (BS) detector receive the refracted light with a 45° angle from the incident ray. Every scan produces 1625 results since the transmission data are collected every 40 μm through the full cell height (65 mm). This instrument allows detecting any instability phenomenon occurring in the sample during the storage. In particular, change in the volume fraction of the particles highlights migration phenomenon, while variation in particle diameter reveals coalescence phenomenon; as an output, variation of T and BS signals are obtained. The comparisons between the formulations are based also on Turbiscan Stability Index (TSI), which provides the numerical quantification of the general behavior of the formulation. Based on previous studies, demonstrating the importance of this apparatus in the assessment of the physical stability of

nanoformulations (Carbone et al., 2020; Bonaccorso et al., 2021a, b), a 30-days analysis was carried on at 25.0 ± 1.0 °C on 20 mL of each prepared NLC.

2.2. Biological materials

Three insect species having different feeding strategies, the sap-sucking *A. gossypii,* the chewer *S. littoralis* and the leafminer *T. absoluta*, were used for the experiments. Insect colonies were established in 2008 from individuals collected in protected vegetable crops in Ragusa (Italy) and breed twice a year with new specimens to reduce the genetic drift. Laboratory insect rearings were located in separated chambers at the Department of Agriculture, Food and Environment of the University of Catania (Italy) and kept at controlled environmental conditions, as follows: 24 ± 2 °C, 50 ± 10% R. H., photoperiod of L14: D10 for *A. gossypii* and *T. absoluta*; and L12:D12 for *S. littoralis*. *Aphis gossypii* was maintained on zucchini plants, *Cucurbita pepo* cv "Bianca di Trieste" (Cucurbitaceae), according to Ricupero et al. (2020). *Spodoptera littoralis* was reared on artificial diet in ventilated and screened plastic boxes (10 × 15 × 5 cm) following the methodology proposed by Di Lelio et al. (2019), *Tuta absoluta* was reared on tomato, *Solanum lycopersicum* cv "Creativo" (Solanaceae), as described by Zappalà et al. (2012).

For the experiment, newly molted (12 ± 6 h-old) *A. gossypii* adults were obtained by isolating, seven days before the experiment, coetaneous 3rd instar nymphs from the rearing on clean zucchini pots. *Aphis gossypii* isolated nymphs were kept at the same standardized laboratory conditions. Second instar larvae of *S. littoralis* and *T. absoluta* were isolated from the rearing.

Zucchini plants at the phenological stage of 3rd true leaf grown in 2 L pot were used in the bioassay for *A. gossypii*. Tomato and sweet pepper, *Capsicum annuum* cv "Grossum" (Solanaceae), plants that reached the phenological growth stage of 5th true leaf on main stem unfolded (BBCH-id 105) (Feller et al., 1995), were used in the bioassays for *T. absoluta* and *S. littoralis*, respectively. Plants were grown in squared pots (10 × 10 × 15 cm) with a mixture of topsoil (Gramoflor®, GmbH & Co. KG) and vermiculite (VIC, Italiana®) in greenhouses (200 × 300 × 300 cm) at natural environmental conditions and fertilized with Greenleaf 20.20.20® (Biolchim s.p.a., Italy) every fifteen days.

2.3. Toxicological bioassays

The NLC formulations used throughout the following toxicological bioassays on three target insect pests consisted of NLCs dispersed in distilled water in the ratio 30:70 v/v. Experimental arenas were kept in climatic chambers at the same controlled environment conditions described for the insect laboratory rearing.

2.3.1. Aphis gossypii

The topical contact toxicity of EO-NLCs was evaluated on the survival and the progeny of *A. gossypii* by spraying coetaneous and young (0–2 d old) adults. For this bioassay, each single replicate was represented by twenty adults feeding on a pesticide-free zucchini plant that were sprayed following the methodology described by Sciortino et al. (2021). The survival of *A. gossypii* sprayed individuals was assessed under the binocular after 24 h, dead aphids were considered as such if they did not react when touched with a fine paint brush. The total aphid progeny, i.e., number of produced nymphs by survived females, was counted daily for ten consequent days after the beginning of the EO-NLC exposure. The toxicity of EO-NLCs was compared with an untreated control, i.e., distilled water, the CT-NLC and a treated control (Afidane® 200 SL, Chimiberg, Italy, a.i. imidacloprid, at the label rate of 750 mL/ha recommended against aphids on cucurbit crops). Per each tested EO-NLCs and the controls the bioassay was replicated five times.

2.3.2. Spodoptera littoralis

The toxicity of EO-NLCs was evaluated in terms of lethal and sublethal effects on *S. littoralis* by topical contact and ingestion routes of exposure. The lethal effect was assessed on the larval survival; while the sublethal effects on the feeding activity of survived larvae. For ingestion exposure, the leaves were cut from healthy and pesticide-free sweet pepper plants, and leaf disks with a diameter of 4 cm were made. From a distance of 20 cm each EO-NLC solution (2 mL) was sprayed with a hand-sprayer, as described by Passos et al. (2022), on the total leaf disks area and let dry in laboratory conditions. Sprayed sweet pepper leaf-disks were placed in a plastic box (6 cm diameter and 4 cm height) and five 2nd instar *S. littoralis* larvae were posed directly on the leaf disks allowing them to feed.

For the topical contact exposure, the leaves were cut from healthy and pesticide-free sweet pepper plants, and leaf disks with a diameter of 4 cm were made and placed in the plastic box with the same characteristics of those for ingestion exposure. Five 2nd instar larvae of *S. littoralis* were sprayed with a hand-sprayer from a distance of 20 cm with 2 mL of solutions in an absorbent paper sheet (to prevent larval drowning caused by excess of solution) as described above and then released on leaf-disks allowing them to feed. An untreated control, i.e., distilled water, a treated control (Karate Zeon®, Syngenta Italia S.p.A., a.i. lambda-cyhalothrin, at the label rate of 125 mL/ha recommended against lepidopteran pests on solanaceous crops) and the CT-NLC were included to compare toxicity of EO-NLC solutions. Per each exposure route, each tested EO-NLCs and the controls, the bioassay was replicated five times.

Three days after the exposure beginning, the survival of the larvae was assessed by probing them with a fine paint brush. Moreover, the eroded leaf area (mm^2) by each larva was assessed through the image acquisition process. Leaves were scanned with a high-resolution Epson® Perfection 4180 Photo in JPG format with 800 dpi of resolution. The resulting images were imported in Adobe Photoshop® (Adobe System Inc. 1990–2018) and a scale by a digital ruler was set as 318 pixel = 10 mm. With "Magnetic lasso" tool, the eroded leaf area was manually selected and the data were collected.

2.3.3. Tuta absoluta

EO-NLCs impact on *T. absoluta* was evaluated in terms of survival and feeding activity following topical contact and ingestion exposures. For the ingestion exposure, two 2nd instar larvae of *T. absoluta* were released to feed in a treated tomato leaf placed in a two-cup experimental arena (Biondi et al., 2012). Tomato pesticide-free leaves cut from healthy plants were sprayed with a hand-sprayer from a distance of 20 cm with the EO-NLC solutions (2 mL) and let dry in laboratory conditions. Treated leaves were thus fixed in the arena and two coetaneous 2nd instar *T. absoluta* larvae from the rearing were placed on the leaves with a soft paint brush.

For topical contact exposure, two 2nd instar larvae of *T. absoluta* were sprayed with the EO-NLC solutions (2 mL) through a hand sprayer on absorbent paper sheet, thus were moved with a paint brush onto fresh and untreated tomato leaves cut from healthy plants previously placed in the two-cup experimental arena.

For both the exposure routes, the larval survival was recorded 72 h after the exposure beginning and the feeding activity was assessed by measuring the eroded leaf area as described in the Section 2.3.2 *Spodoptera littoralis.* An untreated control, i.e., distilled water, a control treated with a commercial insecticide of known efficacy in controlling *T. absoluta* (Steward®, FMC Agro Italia Srl., a.i. indoxacarb, at the label rate of 125 g/ha recommended against lepidopteran pests on solanaceous crops) and the CT-NLC were included to compare the toxicity of EO-NLC solutions. Per each exposure route, each tested EO-NLCs and the controls, the bioassay was replicated five times.

2.4. Data analyses

The data on lethal and sublethal effects of each EO-NLC formulation and control treatments were evaluated on the survival of the three target

species, on the progeny of *A. gossypii* and on the feeding activity of *S. littoralis* and *T. absoluta*. Dataset of dependent variables was checked for homogeneity and normality of variance through Levene and Shapiro-Wilk tests and log-transformed if needed.

The progeny, the survival and the feeding activity of larvae for each dataset independently was subjected to one-way ANOVA analyses and the post hoc analyses were carried out using LSD test ($p \leq 0.05$). Statistical analysis was conducted on R (Version 1.1.463 – 2009–2018, Inc.).

3. Results

3.1. EO-NLC characterization

After 24 h from the preparation, the obtained NLCs (Table 1) were characterized in terms of mean particle size and polydispersity index using PCS. The obtained results, reported in Table 2, show the presence of the EOs in NLC formulation allowed obtaining smaller nanoparticles compared to control CT-NLC. In particular, all EO-NLCs showed the presence of homogeneous nanoparticles of about 200 nm, with a single peak in size distribution as confirmed by PDI values lower than 0.3. ZP results highlight the presence of a negative superficial charge in all the samples, with similar values for Lavender and Peppermint NLCs, and for Rosemary and CT-NCLs. On the other hand, CT-NLC showed PDI values > 0.3 suggesting a low level of homogeneity. As expected, a certain loss due to EOs volatility was observed, even if high EE% values were obtained in the scale L-NLC (EE% 77.65%) > P-NLC (EE% 71.05%) > R-NLC (69.04%). For this reason, each system was further investigated after the centrifugation step.

All NLCs were stored in Turbiscan® at 25 °C for 30 days. The destabilization kinetics showed a significantly different stability between control formulation CT-NLC and EO-loaded NLCs: after 4 weeks of storage, TSI value for CT-NLC are about 26-fold increased, while for L-NLC and P-NLC are about 15-fold increased (Fig. 1). Based on TSI profiles reported in Fig. 1, the most stable formulation results to be R-NLC. In particular, the following stability scale could be defined: R-NLC > L-NLC > P-NLC > CT-NLC.

From backscattering profiles, it is also possible to establish the type of instability occurring in the formulations (Fig. 2). In particular, all the samples showed a migration of the nanoparticles to the bottom of the cuvette, which is slight for EO-NLCs and emphatic for CT-NLC. However, for EO-NLCs this phenomenon is not significant since the variation in backscattering (ΔBS) was lower than 15%, while for CT-NLC it emerged also a sedimentation phenomenon associated to particles aggregation (ΔBS > 20%) as predicted by the high PDI value.

Detailed results obtained from destabilization kinetics analysis (TSI values), reported in Fig. 1, were in line with the affirmations above. In particular, in the first few days some similar instability phenomenon occurred in L-NLC and P-NLC, contrarily from R-NLC. After 1 week of storage until 3 weeks, L-NLC and R-NLC behaviors were quite similar, while a significant particle migration to the bottom of the cuvette was detected for P-NLC. During the last week, L-NLC showed a great increase in TSI value reaching P-NLC curve, while R-NLC profile remained quite stable.

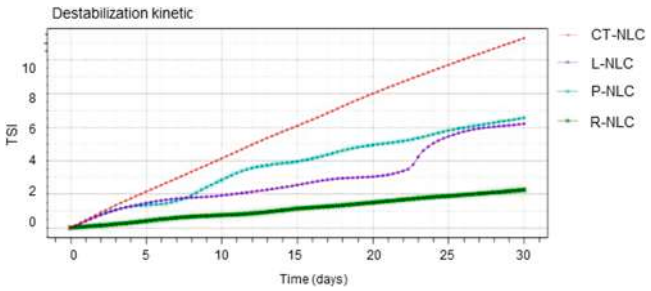

Fig. 1. Destabilization kinetics represented in terms of evolution of Turbiscan® Stability Index (TSI) of NLCs prepared with Lavender, Peppermint or Rosemary stored 30 days at 25 ± 0.5 °C.

Table 2
Mean ± SD particle size (Zave, nm) and Polydispersity Index (PDI) of the prepared Lavender NLC (L-NLC), Peppermint NLC (P-NLC) and Rosemary NLC (R-NLC).

Sample	Zave ± SD (nm)	PDI ± SD	ZP ± SD (mV)
L-NLC	198.9 ± 12.3 *	0.216 ± 0.009 *	-10.8 ± 1.3
P-NLC	179.85 ± 7.4 *	0.207 ± 0.028 *	-10.8 ± 0.9
R-NLC	156.0 ± 1.4 *	0.251 ± 0.026 *	-15.2 ± 0.8
CT-NLC	284.5 ± 59.3	0.480 ± 0.085	-15.4 ± 2.1

* Significance for $p < 0.05$, comparison between EO-loaded NLC (L-NLC, P-NLC and R-NLC) and the control NLC (CT-NLC).

3.2. Toxicological bioassays

3.2.1. Aphis gossypii

NLCs with Rosemary, Lavender and Peppermint EOs caused a significant high mortality by topical contact exposure on *A. gossypii*, in comparison to the untreated control ($F_{5,30} = 62.16$, $p < 0.001$) (Fig. 3A). Also, the progeny of *A. gossypii* females after topical contact exposure with EO-NLCs decreased significantly in comparison to the untreated control ($F_{5,30} = 12.94$, $p < 0.001$) (Fig. 3B). Interestingly, CT-NLC alone caused significant mortality and decreased the offspring in *A. gossypii* similarly to the treated control (Afidane® 200 SL a.i. imidacloprid).

3.2.2. Spodoptera littoralis

3.2.2.1. Ingestion exposure. The tested substances ingested by *S. littoralis* larvae significantly influenced the pest survival in comparison to the untreated control ($F_{5,30} = 8.09$; $p < 0.001$). However, only the survival of the larvae fed on leaves treated with the synthetic pyrethroid (treated control) was significantly lower than all other treatments (Fig. 4A). The feeding activity of *S. littoralis* was significantly affected by the EO-NLCs ($F_{5,30} = 18.36$; $p < 0.001$) (Fig. 4B). Both Lavender and Rosemary NLC-EOs significantly reduced the ingested leaf area by larvae similarly to the treated control, i.e., lambda cyhalothrin. Interestingly, NLC with peppermint EO almost doubled the feeding activity of larvae (≈100 mm^2) in comparison to the untreated control and the CT-NLC.

3.2.2.2. Topical contact exposure. The effect of the topical contact exposure on the survival of *S. littoralis* larvae was significant ($F_{5,30} = 5.21$; $p = 0.002$); however, only the treated control, i.e., lambda cyhalothrin, caused a significantly lower survival of the exposed insects (Fig. 5A). The eroded leaf area by *S. littoralis* larvae after topical contact exposure to EO-NLCs was significantly influenced by the EO-NLC treatment ($F_{5,30} = 10.62$; $p < 0.001$). CT-NLC increased S. *littoralis* larvae feeding activity (≈90 mm^2), although this difference was not significant compared to the untreated control (Fig. 5B). Only *S. littoralis* larvae topically sprayed with the lambda cyhalothrin, i.e., treated control, as expected, showed neither survival nor feeding activity, resulting both the observed parameters equal to 0%.

3.2.3. Tuta absoluta

3.2.3.1. Ingestion exposure. NLCs with Rosemary, Lavender and Peppermint EOs had no significant influence on the survival of *T. absoluta* larvae by ingestion exposure ($F_{5,30} = 1.70$; $p = 0.17$) (Fig. 6A). The eroded leaf area by *T. absoluta* larvae after leaf exposure to EO-NLCs was not significantly influenced by the EO-NLC treatment

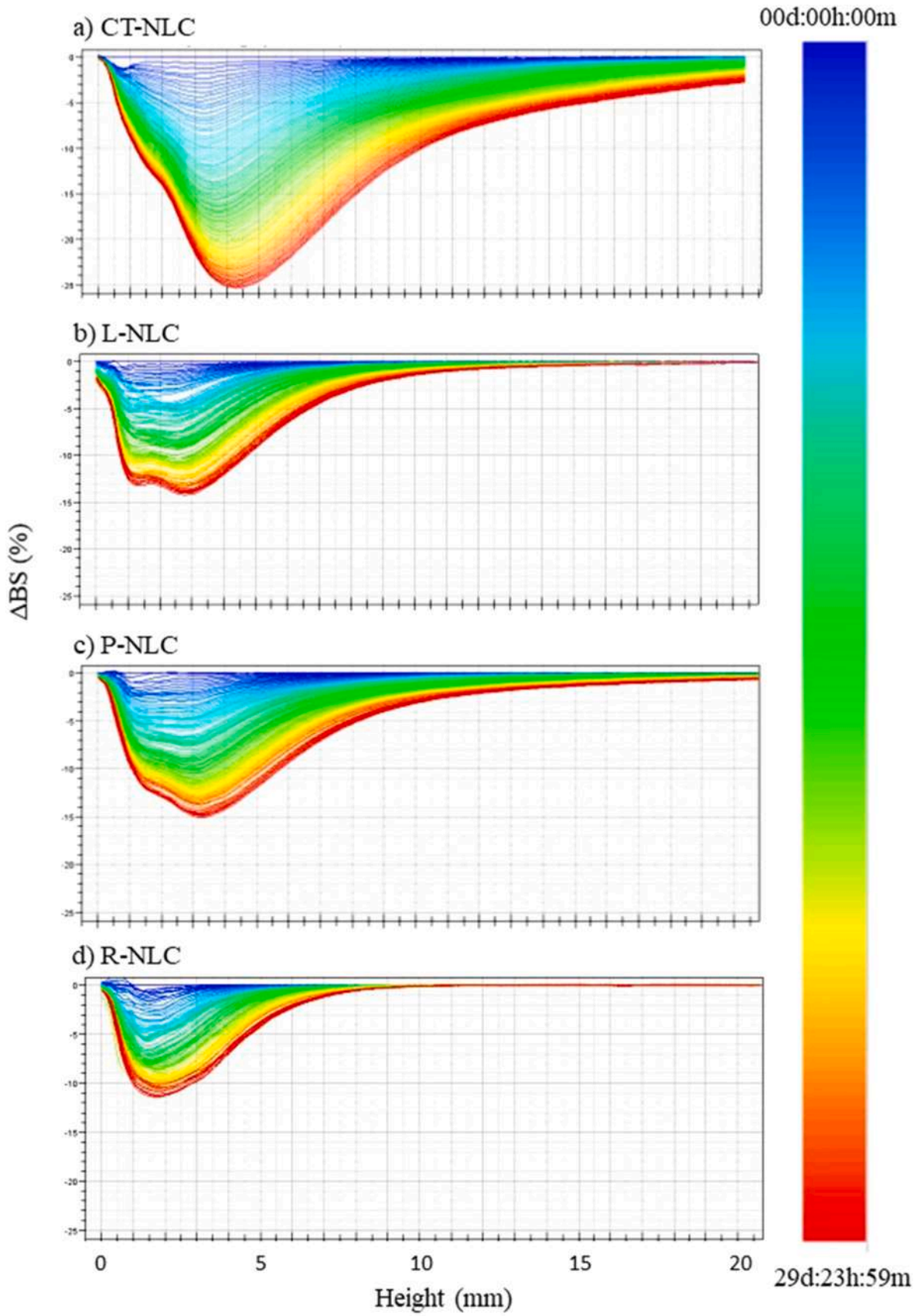

Fig. 2. Backscattering profiles (ΔBS) of CT-NLC (a), L-NLC (b), P-NLC (c) and R-NLC (d) stored in Turbiscan® for 30 days at a temperature of 25.0 ± 1.0 °C. Data are presented as a function of time (0–30 days) of sample height (0–20 mm) (the direction of analysis time is indicated by the arrow).

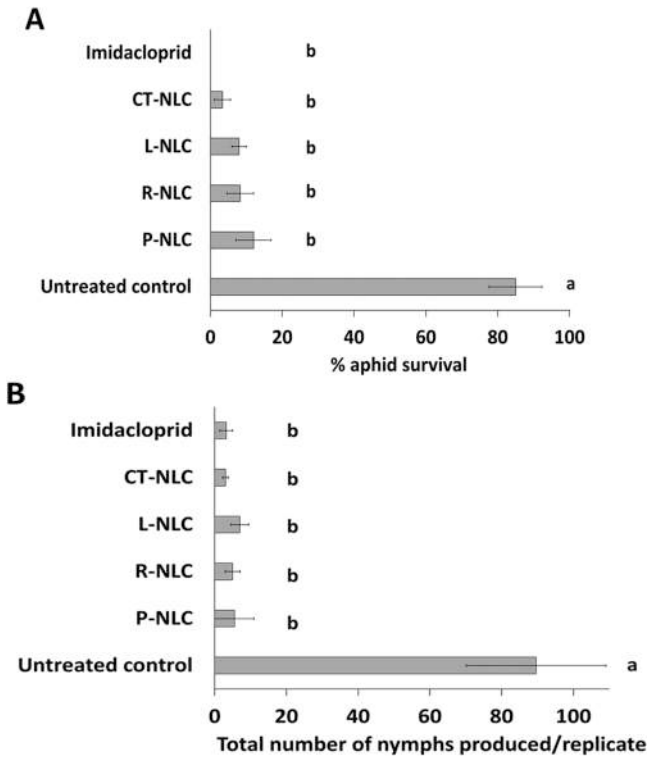

Fig. 3. Mean (± SE) aphid survival (A) and mean (± SE) progeny (B) of *Aphis gossypii* adults topically exposed to EO-NLCs, control NLC and treated control with a commercial synthetic insecticide. Bars with different letters indicate significant differences at $p < 0.05$ (ANOVA, LSD post hoc test).

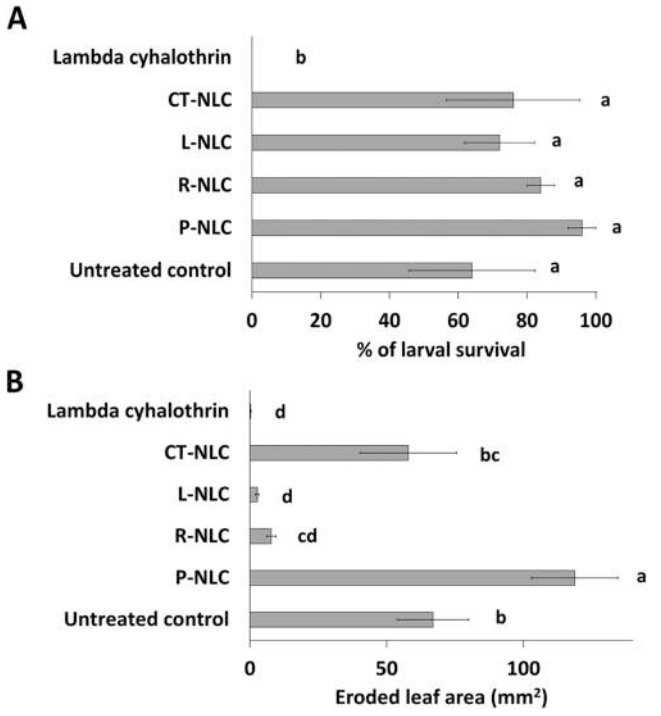

Fig. 4. Mean (± SE) larval survival (A) and mean (± SE) eroded leaf area (mm^2) (B) of *Spodoptera littoralis* larvae on sprayed leaves with EO-NLCs, control NLC and treated control with a commercial synthetic insecticide. Bars with different letters indicate significant differences at $p < 0.05$ (ANOVA, LSD post hoc test).

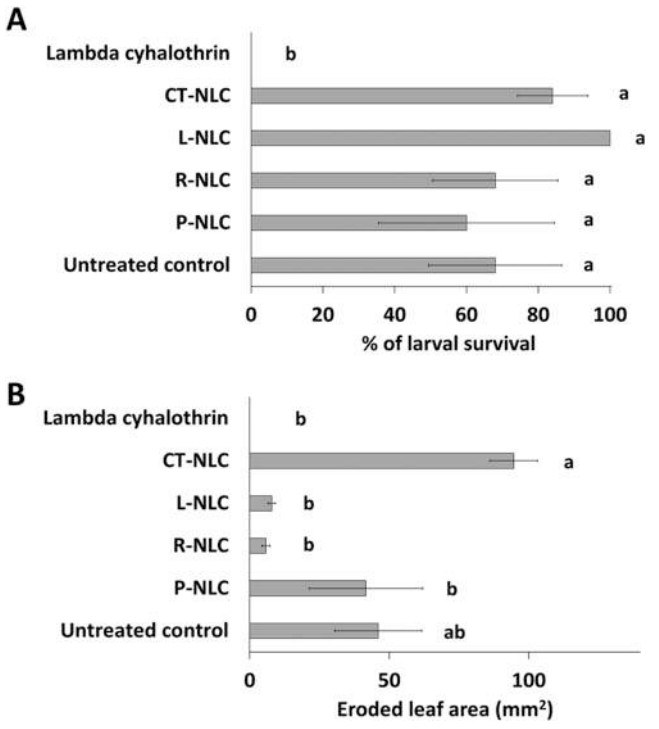

Fig. 5. Mean (± SE) larval survival (A) and mean (± SE) values of eroded leaf area (mm^2) (B) of *Spodoptera littoralis* larvae sprayed topically with EO-NLCs, control NLC and treated control with a commercial synthetic insecticide. Bars with different letters indicate significant differences at $p < 0.05$ (ANOVA, LSD post hoc test).

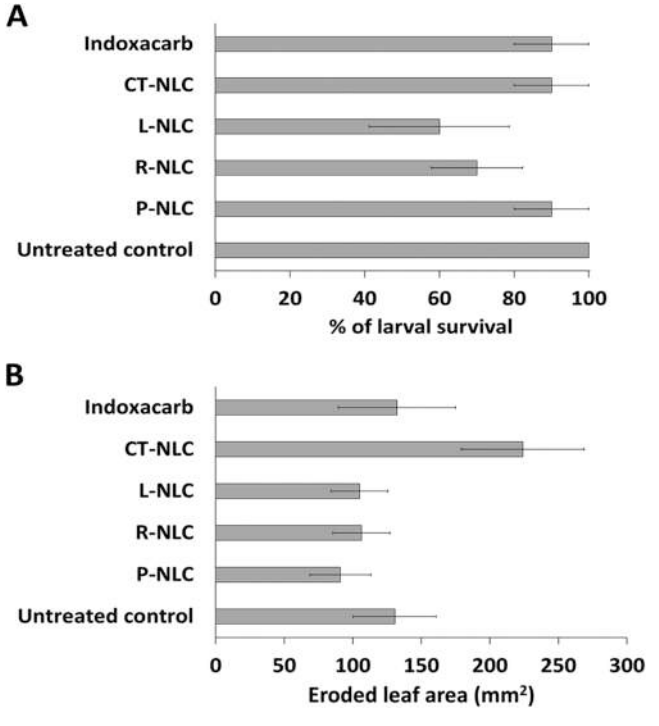

Fig. 6. Mean (± SE) larval survival (A) and mean (± SE) values of eroded leaf area (mm^2) (B) of *Tuta absoluta* larvae on sprayed leaves with EO-NLCs, control NLC and treated control with a commercial synthetic insecticide.

($F_{5,30} = 2.27$; $p = 0.08$) (Fig. 6B).

3.2.3.2. Topical contact exposure. NLCs with Rosemary, Lavender and Peppermint EOs by topical contact exposure did not affect the survival of *T. absoluta* larvae ($F_{5,30} = 1.85$; $p = 0.14$) (Fig. 7A). However, L-NLC and the CT-NLC reduced the larval survival, even if not significantly, up to the ≈ 30% and ≈ 40%, respectively. Also, the eroded leaf area by *T. absoluta* after topical contact exposure to EO-NLCs was not significantly influenced by the EO-NLC treatment ($F_{5,30} = 1.31$; $p = 0.29$) (Fig. 7B). Nonetheless, Lavender EO reduced the feeding activity of *T. absoluta*, but not significantly.

4. Discussion

The results of this study confirm that EOs could be delivered by NLCs with stable and homogeneous nanoparticles. Such new delivery system could represent an innovative and sustainable tool for using bio-active compounds as effective bio-pesticide against insect pests. Moreover, all the EO-NLCs are not phytotoxic for all the species used in the laboratory experiments, this represents an important aspect to evaluate the feasibility of this formulation in field conditions. All EO-NLCs have homogeneous nanoparticles, in terms of size distribution and stability. This suggests that Lavender, Peppermint and Rosemary EOs are able to provide a better organization of raw materials at the nanoparticles interface therefore inducing the formation of nanosuspensions with greater homogeneity. This could be due to the ability of the EOs in altering the crystallinity of the lipid matrix creating a more disordered structure compared to the commercial CT oil (da Silva Santos et al., 2019). However, further studies need to be performed to confirm this hypothesis.

Moreover, ZP results highlight the low aggregation phenomenon due to the presence of a negative superficial charge in all the samples above all for R-NLC and CT-NLC that are more promising in terms of long-term stability. As described by Bashiri et al. (2020), the higher negatively-charged surface compared with lower positively-charged surface developing a high potential difference provides stronger electrostatic repulsion which lead to better stability with lower aggregation. On the other hand, homogeneity has also to be considered because it plays a key role in determining stability. Indeed, CT-NLC that showed a PDI values > 0.3, with low level of homogeneity and with the presence of bigger particles aggregation, could be subjected to possible instability phenomenon (Danaei et al., 2018). For the other EOs, considering the first 3 weeks storage, the good stability of R-NLC and L-NLC could be related to the presence of smaller particles and great homogeneity compared to P-NLC. Nevertheless, values of PDI and particles aggregation of CT-NLC showed higher sedimentation phenomenon than for the other EO-NLCs. As previously reported by Carbone et al. (2018), the phase separation which occurs during the storage is reversible, since it is possible to resuspend the nanoparticles through agitation.

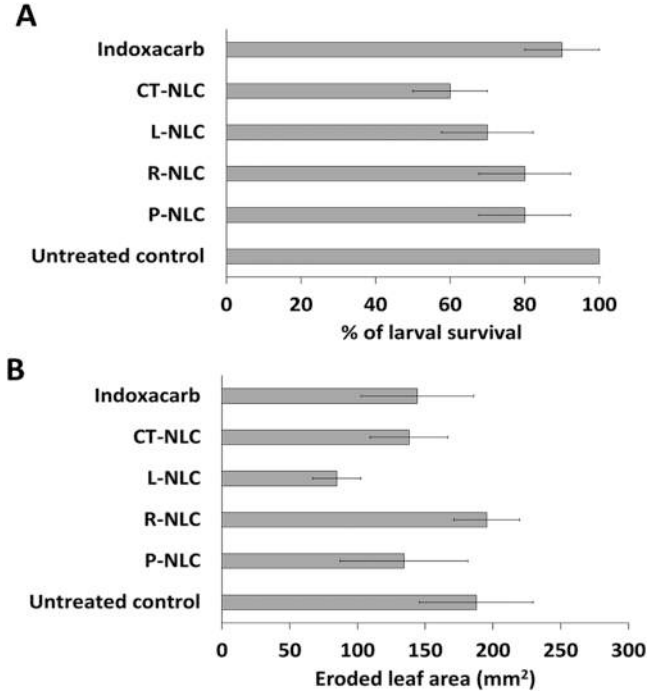

Fig. 7. Mean (± SE) larval survival (A) and mean (± SE) values of eroded leaf area (mm^2) (B) of *Tuta absoluta* larvae sprayed topically with EO-NLCs, control NLC and treated control with a commercial synthetic insecticide.

The results on NLCs toxicological properties on pests showed that CT-NLC alone caused significant mortality and decreased the offspring in the tested aphid pest *A. gossypii*. This suggest that the high mortality and the reduction of aphid fertility can be due to the developed CT-NLC formulation that includes surfactants as main components. Indeed, the toxicity of surfactants against aphid pests is supported by different studies (Wolfenbarger et al., 1967; Imai et al., 1994; Wood et al., 1997) because they penetrate insect cuticle by disrupting cell membranes, causing thereby the desiccation of soft-bodied insects (Kraiss and Cullen, 2014).

For the reduction in *S. littoralis* feeding activity recorded in both the tested exposure routes, i.e., ingestion and topical contact, for Lavender and Rosemary NLCs might be due to the intrinsic toxicity of EOs as previously demonstrated on *Spodoptera* spp. (Ortiz de Elguea Culebras et al., 2018; Sombra et al., 2020). Singular and/or combined EO components, among terpenes and monoterpenes, play a significant role in pest control (Regnault-Roger et al., 2012). Moreover, neither acute toxicity nor a reduction in the feeding activity was caused by EO-NLCs against *T. absoluta* in both the exposure routes, i.e., ingestion and topical contact. The low mortality and the high eroded leaf area observed in *T. absoluta* larvae after ingestion exposure might be explained by the reduced capacity of EO-NLCs to penetrate plant foliar tissue and by *T. absoluta* feeding strategy (Campolo et al., 2017). We also tested the potential topical toxicity of EO-NLCs by exposing young larvae of *T. absoluta*. Although *T. absoluta* larvae penetrate into tomato leaves, stems or fruits (Desneux et al., 2010), they are also able to move actively within aerial plant parts that result more suitable to feed (Torres et al., 2001). Consequently, larvae could be easily targeted by chemicals when foraging outside the mesophyll. Topical exposure of *T. absoluta* larvae has been used to screen the toxicological properties of novel substances (Benchaabane et al., 2016; De Smedt et al., 2016; Rahmani and Bandani, 2021). Indeed, our results showed a low topical toxicity on larvae after topical EO-NLC application can be due to the low capacity of EO-NLCs to penetrate the insect body and the potential combined ability of larvae to detoxify the nanocarried EOs (Ghanim and Abdel Ghani, 2014). Although NLC technology has been used for encapsulating synthetic insecticides (Maroofpour et al., 2021), no information is currently available on the use of this nanotechnology in EO delivery for pest control. To the best of our knowledge, this is the first attempt in using such a promising technology in applying EOs for controlling three economic relevant insect pests having different feeding strategies.

5. Conclusions

Our results demonstrate that EOs nanoencapsulation allowed obtaining stable colloidal formulations characterized by homogeneous small particles, with a mean diameter lower than 200 nm for all the selected EO. The nanosuspensions were able to encapsulate a great amount of EO (≥70%) maintaining a promising long term physical stability, especially in case of R-NLC.

The developed EO-NLCs proved to have some potential as starting point for the development of new plant protection tool as alternative

sustainable tools for topical treatment of insect pests. In summary, for *A. gossypii* all the tested EO-NLCs, including NLC as such, caused high mortality and significantly reduced its progeny by topical contact exposure. Similarly, for *S. littoralis*, Lavender and Rosemary EO-NLCs decreased the feeding activity but not the survival. Conversely, for *T. absoluta* larvae feeding inside tomato leaves Lavender, Peppermint and Rosemary EO-NLCs did not decrease the survival and the feeding activity. Therefore, to obtain encouraging results it is probably necessary to increase the v/v of EOs in NLCs.

Our findings suggest that NLCs can successfully deliver EOs and keep their properties unaltered without penetrating plant tissues, as suggested by the results observed on *T. absoluta*. Nevertheless, the outcomes of the present work need to be supported by further studies on EOs and NLCs, focusing on their delivery dynamics within plant and insect tissue (Athanassiou et al., 2018; Karny et al., 2018). Moreover, increased EO toxicity could be explored by combining multiple EOs in the same formulation (Kim et al., 2021). The bioactivity of these EOs need to be explored thoroughly also towards non-target organisms for evaluating the environmental risk assessment of these potential bio-rational insecticides (Campolo et al., 2020b; Pavela et al., 2020).

CRediT authorship contribution statement

S. Tortorici: Conceptualization, Methodology, Investigation, Data curation, Formal analysis, Writing – original draft. **C. Cimino**: Methodology, Investigation, Data curation, Formal analysis, Writing – review & editing. **M. Ricupero**: Conceptualization, Methodology, Investigation, Data curation, Formal analysis, Writing – review & editing. **T. Musumeci**: Conceptualization, Methodology, Investigation, Data curation, Formal analysis, Writing – review & editing. **A. Biondi**: Conceptualization, Validation, Supervision, Writing – review & editing, Project administration, Funding acquisition. **G. Siscaro**: Conceptualization, Validation, Supervision. **C. Carbone**: Conceptualization, Validation, Supervision, Writing – review & editing, Project administration. **L. Zappalà**: Conceptualization, Validation, Supervision, Writing – review & editing, Project administration, Funding acquisition.

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.

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